Discovery of Bcl-3 inhibitors for the potential treatment of metastatic breast cancer

A thesis submitted *in accordance with the conditions governing candidates* for the degree
of
Philosophiae Doctor in Cardiff University

by

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“Life is like riding a bicycle.
To keep your balance,
you must keep moving.”

Albert Einstein

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Abstract

In HER-2 positive metastatic breast cancer, the proto-oncogene Bcl-3 was found to be over-expressed. Bcl-3 acts as a transcriptional co-activator: it forms a ternary complex with DNA and homodimer $(p50)_2$ and stimulates the transcription of a different panel of genes in the metastatic progression of the breast cancer. Its exact role in endogenous tumours is still unknown. *In vivo* knockdown studies shown, that Bcl-3 deficiency did not affect the primary tumour, but it reduced the occurrence of metastases by 80% without any effects on the normal mammary gland function. Patients with this type of tumour have a poor prognosis, do not respond to the typical treatment or show resistance and side effects to the usual therapeutic options. Hence, there is an urgent need to find new candidate drugs.

A virtual screening targeted against a newly identified Bcl-3 binding pocket allowed the identification of 10 molecules, tested *in vitro* cell based assays. Four molecules were active.

In this thesis, different analogues of these four hit compounds were synthesised. Based on docking studies, another two scaffolds were designed. The non-cytotoxic profile of the new analogues was assessed using the cell titer blue assay. The activity of the compounds was established using the colony forming assay (CFA). To prove the Bcl-3/p50 interaction, immunoprecipitation and co-immunoprecipitation were performed. None of the compounds was cytotoxic. Some of them exhibited a promising activity in the CFA. One molecule exhibited an interesting activity profile both *in vitro* and *in vivo* and in pharmacokinetic studies and it has been forwarded into full pre-clinical development.

List of abbreviations

9-BBN	9-borabicyclo(3.3.1)nonane
Ab	antibody
AcOEt	ethyl acetate
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ASR	age-standardised rate
BBr_3	boron tribromide
Boc	tert-butyloxycarbonyl protecting group
BSA	bovine serum albumin
CFA	colony forming assay
CTB	cell titer blue
Co-IP	co-immunoprecipitation
Cys	cysteine
DCM	dichloromethane
DMC	2-chloro-1,3-dimethylimidazolinium chloride
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethyl-formamide
DMSO	dimethyl sulfoxide
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
Et_3N	triethylamine
EtOH	ethanol
ELISA	enzyme-linked immunosorbent assay
FDA	food and drug administration
HATU	1-[bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HCl	hydrochloric acid
HER2	human epidermal growth factor receptor 2
HOBT	hydroxybenzotriazole
EDCI	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EGFR	epidermal growth factor receptor
EtOH	ethanol

Gln	glutamine
Glu	glutamic acid
Gly	glycine
GST	glutathione S-transferases
HATU	1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
H_2	molecular hydrogen
HCl	hydrochloric acid
His	histidine
Hz	Hertz
IC_{50}	half maximal inhibitory concentration
Ile	isoleucine
IκB	inhibitors of NF-κB
IP	immunoprecipitation
K_2CO_3	potassium carbonate
Leu	leucine
$LiAlH_4$	lithium aluminium hydride
Lys	lysine
LoD	limit of detection
LoQ	limit of quantification
LPS	lypopolysaccharide
MAPK	mitogen - activated protein kinase
mCPBA	meta-chloroperoxybenzoic acid
Met	methionine
$MgSO_4$	magnesium sulfate
mL	milliliter
mmol	millimol
MMP	matrix metalloproteinase
MOE	molecular operating environment
MOM	chloro methyl methyl ether
mTOR	mammalian target of rapamycin
MeOH	methanol
MW	molecular weight
Na_2CO_3	sodium carbonate

NaOH	sodium hydroxide
NBS	*N*-Bromosuccinimide
NEMO	NF - κB essential modifier
NF-κB	nuclear factor binding to the intronic kappa-light-chain enhancer element in B-cells
NIK	NF-κB inducing kinase
NLS	nuclear localisation sequence
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline with Tween 20
PDB	protein data bank
Pd/C	palladium on carbon
Phe	phenylalanine
PI3K	phosphatidylinositol 3-kinase
pNPP	para - nitrophenylphosphate
PPIs	protein - protein interactions
ppm	parts per million
Pro	proline
RLU	relative light units
RTK	receptor tyrosine kinase
SD	standard deviation
Ser	serine
SERM	selective oestrogen receptor modulator
$SOCl_2$	thionyl chloride
STAT	signal transducer and activator of transcription
TAD	transactivation domain
TBS	tris-buffered saline
TBS/T	tris-buffered saline with tween 20
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
t-BuLi	tert-butyllithium
TEA	triethylamine
TFA	trifluoro acetic acid
THF	tetrahydrofurane
Thr	threonine
TNF-α	tumour necrosis factor-α
Trp	tryptophan

Tyr	tyrosine
SEM	standard error of the mean
SN_2	nucleophilic substitution 2
$SnCl_2$	tin (II) chloride
Val	valine
vHTS	virtual high throughput screening process
WT	wild type

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Section 1:

INTRODUCTION

1.1 Breast cancer

Nowadays, breast cancer is the most common cancer in women worldwide and the foremost cause of death in females in comparison to other types of cancer.[1] Prevailing in more than 151 countries,[2] around 1.68 million new patients are diagnosed with breast cancer every year, comprising approximately 25% of all female cancers.[3]

Breast cancer occurs in both developed and developing countries; according to the Globocan estimation project, in 2012 Europe was the continent with the highest incidence, followed by North America, Western Pacific regions, South East Asia and East Mediterranean regions as depicted in Figure 1.[4]

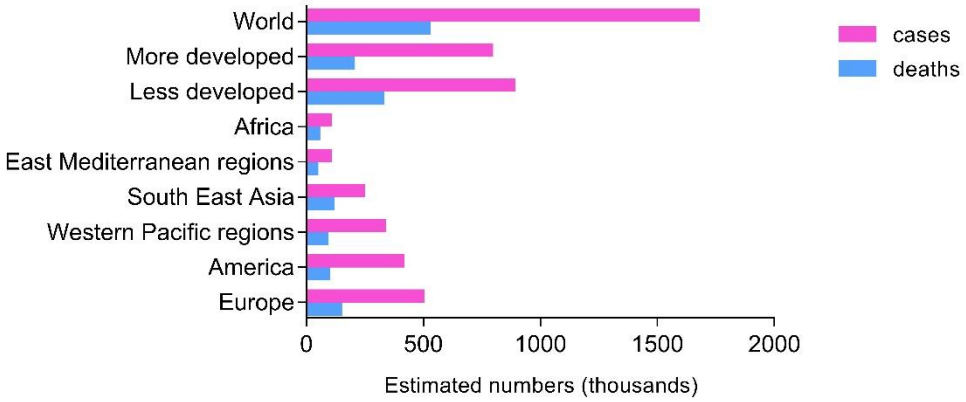


Figure 1. Estimated numbers of cases and deaths of breast cancer worldwide in 2012.

Incidence and mortality rates were highest in Northern Europe, notably in the United Kingdom,[5] which shows the sixth highest world age-standardised incidence rate (approximately 121 cases per 100 000).[6]

In the United Kingdom alone, over a period of 40 years from 1971 to 2010, cancer morbidity has increased steadily: in 2010 the age-standardised incidence rate was 126 per 100 000 women, while it was only 66 per 100 000 women in 1971. Focusing on the mortality rates, over the same period of time, a decrease was reported: in 2011 the mortality rate for breast cancer was 24 per 100 000 women compared to 39 in 1971.[7]

In 2013, as represented in Figure 2, breast cancer was the most common malignancy in the United Kingdom with 60693 new cases: 31% of all female cancers and 0.5% of all male cancers. The lifetime risk of a woman developing breast cancer is estimated to be one in eight nowadays, while the lifetime risk of a man is one in 870.[8]

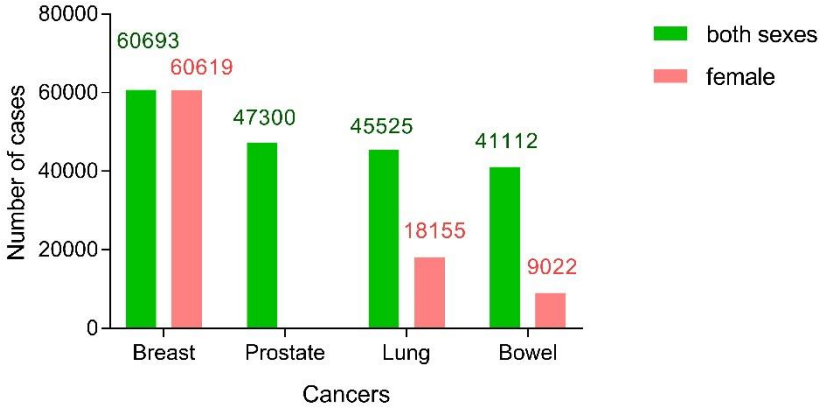


Figure 2. Incidence of the top 5 rank cancers in the United Kingdom (2013).

An analysis of the incidence of breast cancer and *in situ* breast cancer (see section 1.2) in women in the four countries of the United Kingdom is reported in Figure 3: a lower percentage of patients is affected by *in situ* disease. Breast cancer exhibited the highest incidence in England with the 84% of new cases, while only 2% of cases were diagnosed in Northern Ireland.[9]

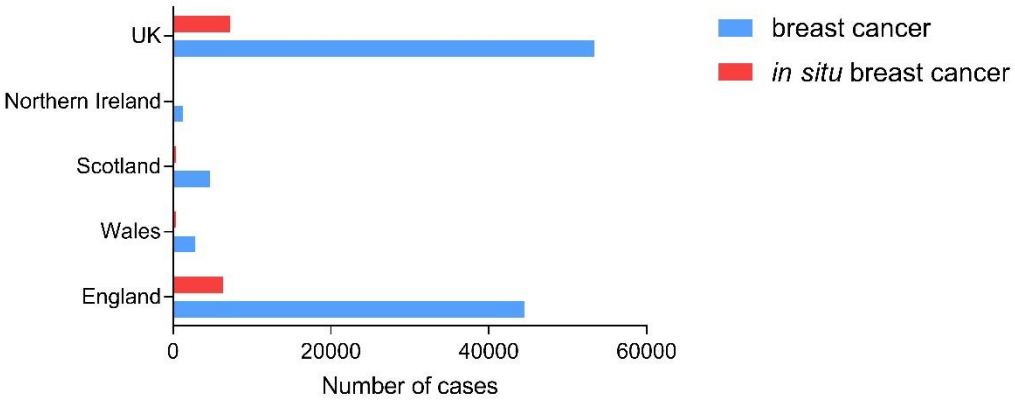


Figure 3. Breast cancer and *in* situ breast cancer number of cases by country in the United Kingdom in 2013.

Over the last decade, a 41% increase in the morbidity data for breast carcinoma in female patients has been registered in the United Kingdom.[10]

The introduction of screening programs (mammography or magnetic resonance imaging) supports early detection of localised or *in situ* disease, a largely curable condition.[11] Earlier diagnosis and screening programs individualise clinicopathological features and prognostic factors (breast cancer staging, genetic mutation and molecular subtypes of breast cancer, section 1.2, 1.3) to make treatments more likely effective (discussion of adjuvant and neo-adjuvant therapy in section 1.5).[12]

The situation is different for those breast cancer patients who develop clinically detectable metastases.[13] The invasive form of breast cancer accounts for approximately 75% of all breast cancer diagnoses.[14] Approximately 6-10% of newly diagnosed breast cancers are initially metastatic forms; 20-30% of the existing breast cancer evolves to metastatic form as recurrence after prior treatment of early stage disease occurs. In addition, more than 50% of recurrences occur 5 years after diagnosis, also many are reported after 10 or 20 years.[15] The lowest 5-year survival rate, 16%, is associated with the most advanced and aggressive stage of metastatic breast cancer (stage IV), as reported in Figure 4. By contrast, 5-year survival of early-stage cancers is 84% (stage I).[16]

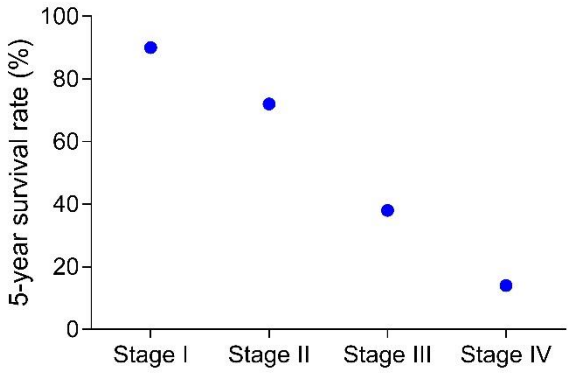


Figure 4. Correlation between pathological tumour staging (section 1.2) for invasive breast cancer and 5-year survival rate.

In the metastatic setting, new therapies are increasingly becoming available. However, even the use of targeted drugs, novel single therapeutic agents or combination of chemotherapeutic regimes, has led only to a slight improvement in survival rates. Thus a complete cure for patients with metastatic breast cancer remains a hope for the future because of resistance to drugs, the need for tailored therapy depending on prognostic factors, side effects and lastly the expensive cost of treatment. Despite the improvement in the diagnosis and treatment, breast cancer remains the most common cause of death in women worldwide.[17]

1.2 Stages of breast cancer

Breast cancer is a heterogeneous, malignant epithelial tumour originating from breast tissue. It is known that there are five stages of breast cancer according to the anatomic and prognostic classification.

Stage 0 is characterised by the development of abnormal cells, not yet invasive. The cancer cells are present in the lining of the breast duct, but they have not yet invaded the breast tissue and they do not have invasive features. It is a ductal carcinoma in situ (DCIS). It is a largely curable condition by total mastectomy or lumpectomy.[18]

Stage I is the early stage of breast cancer: breast cancer has invaded beyond the duct lining, but not beyond the breast. The lymph nodes are not involved at all (stage IA) or a small group of cancer cells (0.2 mm - 2 mm) are present also in the lymph nodes (stage IB). Because of the size of the tumour, it is surgically operable (breast-conserving surgery). Other therapeutic options include radiation and adjuvant systemic therapy (hormone therapy or aromatase inhibitor, given after surgery) to lower the probability of recurrence, although it is not common and the overall survival rate is 84%, as shown in Figure 4.[19]

Stage II is characterised by metastases in the lymph nodes or by the presence of a tumour, which is not larger than 5 cm. The lymph nodes are involved: cancer has started to spread to the auxiliary lymph nodes. In this stage, it is possible to resort to systemic therapies before surgery (neo-adjuvant) and after surgery. If possible, breast conservation surgery is the choice. The average survival is approximately 73% after five years.[20]

Stage III is named also "locally advanced breast cancer": the primary tumour is larger than 5 cm in diameter without metastases or it is between 2 and 5 cm in diameter, with evident metastasis. In the first case, the treatment involves only surgery (mastectomy); while, in the second case, neo-adjuvant and adjuvant therapies support surgery and radiation. When cancer has started to spread, the first locations are the auxiliary lymph nodes or the lymph nodes near the breastbone. The overall survival rate is significantly lower, 38%, than in the previous stage.[21]

Stage IV is known as "advanced"; it is the one in which metastasis are present in other parts of the body (liver or bones). Local treatment, such as mastectomy or lumpectomy or radiation, may not be used. Hormone therapy, chemotherapy and targeted drugs as single agent or in combinations are used. It is the most aggressive stage and the long-term survival rate is the lowest: less than 16% of patients affected by this aggressive form of breast cancer will survive for 5 years.[18]

1.3 Microarray-based breast cancer subtypes

Recently, microarray-based gene expression assays have revealed that breast cancer is a heterogeneous disease. Depending on clinical-pathological factors, it is possible to classify the human breast tumours into six common types: Luminal A, Luminal B, Her-2-like, Basal-like, Claudin low, Normal-like.[22] Each of these subtypes is different not only in terms of prognostic factors but also of

the likelihood of response to treatments. Breast cancer cells may or may not express three different kinds of receptors on their surface and in their cytoplasm and/or nucleoplasm: the oestrogen receptor (ER), the progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her-2). Cancer cells expressing the oestrogen receptors are named ER+ and their growth depends on oestrogen. Similarly, growth of PR+ cancer cells depends on the hormone progesterone. These types of cells may be sensitive to hormonal or anti-oestrogen therapy. Approximately 30% of breast cancers over-express the Her-2 receptor: this tumour is fast growing and more aggressive than the other subtypes of cancers.[23] Due to its important role in the development of metastatic breast cancer, it will be discussed further in section 1.3.1.

The Luminal A subtype is characterised by an ER+ and/or PR+ and Her2- phenotype: it expresses ER-related genes. Usually, it has a favourable prognosis. This subtype of cancer cell responds to endocrine therapy.[24]

The Luminal B subtype has an ER+ and/or PR+ and Her2+ phenotype (triple positive): unfortunately, it is less responsive to endocrine therapy and has a poorer prognosis.[20]

The Her-2-Like phenotype is ER-/PR- and Her+: despite the poor prognosis, it has a high response to cytotoxic chemotherapy. [25]

Tumours characterised by cells that do not express any of these three receptors are named triple negative or Basal-like: they exhibit a stem cell phenotype, acquiring metastatic potential. Poor prognosis and the highest reoccurrence probability are linked to this cancer type.[26]

The Claudin-low subtype exhibits low expressions of those genes involved in the cell-cell interaction and adhesions, of the Her-2 related genes and of hormone-related luminal genes. A more aggressive type of metastatic breast cancer has been found to express the typical features of the Claudin-low subtype: triple negative invasive ductal carcinoma, with a response to chemotherapy that is intermediate between that of basal-like and luminal phenotype tumours.[25]

The Normal-Like phenotype (ER-, PR-, Her-2-,) is characterised by high expression level of genes of normal basal epithelial and adipose cells, and low expression level of those genes involved in the luminal epithelial cells. This breast cancer subtype is triple negative, lacking the usual gene expression of the Basal-Like subtypes and showing a similarity to the normal mammary cells. It has the better prognosis. [27]

1.3.1 HER2 enriched subtype of breast cancer

The HER family of proteins, also termed ErbB, includes four receptor tyrosine kinases (RTK) in humans: Her1 (EGFR, ErbB1), Her2 (Neu, ErbB2), Her3 (ErbB3), and Her4 (ErbB4).[17]

They are expressed in epithelial, mesenchymal and neuronal tissues and involved in several physiological events such as proliferation, differentiation, migration and apoptosis.[28, 29]

In humans, insufficient ErbB signalling correlates with the development of neurodegenerative diseases, such as multiple sclerosis and Alzheimer's disease, while higher ErbB expression level is associated with the development of a wide variety of solid tumours.[29]

These transmembrane tyrosine kinase receptors are structurally related and composed of three main parts: an extracellular region responsible for their activation and dimerisation, a single membrane-spanning region and a cytoplasmic tyrosine kinase domain. The extracellular region is made up of four domains: as a tandem repeat of two domain units (domain I and III) followed by the other two domains (II and IV). Two conformations exist for the domains of ErbB receptors: 1) tethered, which is inactive and 2) extended, which is active. As represented in Figure 5, in the inactive form, domain II and IV make an intra-molecular contact preventing dimerisation. Upon specific ligand binding (TGF-α, epiregulin et al.), a rearrangement occurs, domains I and III are closer and they make domains II available for dimerisation.[17]

Homo- or hetero- dimerisation then leads to catalytic activation of the receptors for their intrinsic kinase activity; this is the starting point for a cascade of events leading to the downstream signalling pathway, which involves a complex machinery and multiplayers, especially due to the involvement of these receptors in the signalling events of different classes of other receptors.[26]

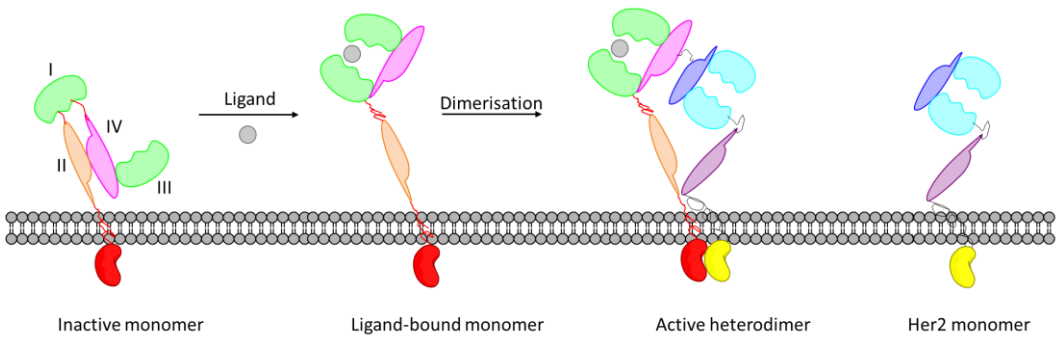


Figure 5. Activation of Her receptors and conformation of Her2 receptor.

Importantly, as represented in Figure 5, Her2 lacks the aforementioned contact between domain II and IV and thus exists always in the active, dimerisation-prone, extended conformation, even in absence of a ligand. In contrast, in absence of their ligands, the other three receptor subtypes are in the inactive tethered conformation.[28] Moreover, Her-2 is an orphan receptor: none of the ligands for the other members binds to Her-2 and no other ligands have been found so far.[26]

Despite the absence of known ligands, Her-2 is the preferred binding partner involved in the dimerisation of the three Her receptors. A possible explanation for this preference could be that, as mentioned before, the extracellular domain of Her-2 is always in an open, thus more readily bondable, conformation.[17]

Activation of Her-1, Her-3, and Her-4 may facilitate transactivation of Her2 through ligand-induced hetero-dimerisation.

Over the last two decades, Her2 receptor has caught a lot of attention due to its leading role in cancer progression.

The receptor's over-expression correlates with several aggressive subtypes of breast cancer.[27] Indeed, expression of the Her2 receptor plays a leading role in 25% of human tumours such as breast cancer, lung or stomach carcinomas.[17]

Her2-overexpressing breast cancers are characterised by a poor prognosis and an increased incidence of metastasis. On the other hand, an endogenous normal level of Her2 is necessary for the development and growth of the normal breast.[29]

The Her2 receptor may activate a plethora of signalling pathways. Among these, the two most important for cancer progression and invasion are the Mitogen-Activated Protein Kinases (MAPK) and the PhosphatidylInositol-4, 5-bisphosphate 3-Kinase - Protein kinase B (PI3K-AKT). Activation of the MAPK pathway leads to increased cellular division and proliferation; while, the homo- or hetero- dimerisation of Her receptors trigger the PI3K-AKT signalling pathway leading to inhibition of apoptosis.[31]

Moreover, the PI3K-AKT pathway is involved in Her-2 mediated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation.

Aberrant activation of the NF-κB signalling pathway has been observed in several types of cancers, including in Her2/neu+ and basal subtype of breast cancer.[32] Indeed, a critical liaison is between Her-2 and NF-κB.[33]

Owing to the importance of Her-2 receptors in human breast cancers, many efforts have been made to develop Her-2 targeted therapeutics, such as monoclonal antibodies or tyrosine kinase inhibitors (see section 1.5.2). Unfortunately, it is worth mentioning that many patients affected by $Her2^+$ cancers do not respond at all or develop resistance to the treatment with these monoclonal antibodies or targeted chemotherapy.[31]

1.4 Metastatic breast cancer

The primary or original breast cancer is localised in breast tissues. However, one out of three women may develop the most aggressive form of breast cancer, characterised by the development of metastases, which may spread to distant sites, such as lungs, liver, brain, bone or regional lymph nodes. The hallmarks of metastases are: loss of cellular adhesion, activation of the epithelial-mesenchymal transition, increased motility and invasiveness, intravasation/extravasation, micrometastasis and organ-specific metastasis.[34]

The formation of metastases is a pathophysiologic mechanism (Figure 6), which is not yet completely clear.

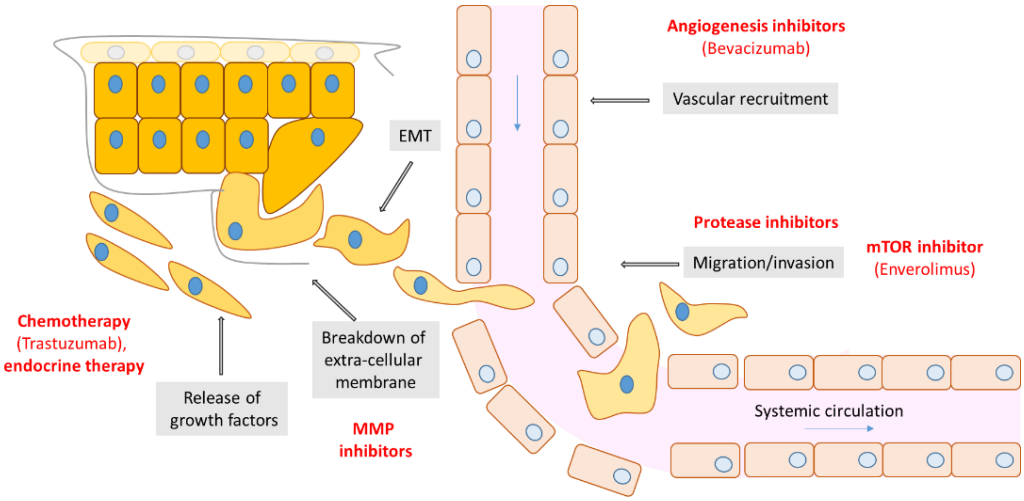


Figure 6. Multistep cancer progression and possible therapeutic options.

A better understanding of the different signalling machinery influencing the metastatic development is necessary and research into these questions is ongoing.[4] Several mouse models have been studied to explain the metastatic process.

It is established that the formation of metastasis is an intrinsic capacity or an acquired feature of the cancer cells of the primary tumours.[35] Metastasis is a heterogeneous multi-step process, consisting of several dynamic interactions between cancer cells and the microenvironment of the affected organ.

In invasive breast cancer, the cancer cells leave the primary site of growth to form secondary tumours in distant sites: the tumour cells must develop new features to carry out the metastatic cascade of events involving the migration, invasion or the colonisation. The primary tumour cells develop an aggressive phenotype, which is characterised by oncogenic mutations and epigenetic plasticity.[36] For example, the most aggressive form of breast cancer is characterised by Her2 over-expression; in these tumours, a high level of active MAPK pathway has been detected, essential for migration.

Additionally, heterodimers with Her-2 may promote activation of PI-3K pathways, a leading player in the migration and cell survival process.[37]

Different factors in the microenvironment may enhance the aggressiveness of primary tumours, such as hypoxia or low pH. Basement membrane or immune surveillance prevent the tumour progression, although on the other hand, they drive the selection of features that enable the cancerous cells to overstep them.[36]

Moreover, the overexpression of anti-apoptotic effectors or loss of caspase-8 expression may inhibit cell death and favour invasion and the metastasis. Once the cancer cells have lost intercellular adhesiveness, the decrease of E-cadherin expression is associated with the progression toward the epithelial to mesenchymal transition (EMT), which facilitates the intravasation and the invasion. Although the EMT is a milestone in different physiological process (gastrulation or heart morphogenesis, for example), recently several studies highlight that the EMT promotes the motility and invasiveness of circulating cancer cells.[6]

During the cell invasion step, neoplastic cells may translocate across extracellular matrix barriers: the tumour cells may migrate away from the primary tumour, entering the lymphatic vessels or the bloodstream.[37] Accumulating evidence suggests that the secretion of proteolytic enzymes is essential in order to degrade extracellular matrix components; a family of these proteolytic enzymes is named matrix metalloproteinases (MMP).[38]

Recently, a new MMP has been discovered: ADAM is a disintegrin and metalloproteinase.[39] Its emerging role in cancer has been highlighted: its protease activity is essential for invasion.[31]

After entering into the circulatory compartment, the malignant cells may survive by co-opting blood platelets, using them as "shields" to overcome the immune-mediated mechanisms of clearance.[40] To colonise distant sites, cancer cells must evade from the endothelial vasculature in the extravasation step using both the pre-existing vasculature, *de novo* vasculature or by lymphangiogenesis. The ErbB receptor may influence the process of the tumour-induced neo-angiogenesis, because of its involvement in the production of pro-angiogenic factors (VEGF is an example).[37]

Primary tumours produce haematopoietic factors (VEGFR1, CD133, CD34) from the bone marrow: their function is to precondition the metastasis sites in order to create a premetastatic niche where the extravasated malignant cells may home and colonise a specific organ.[35]

Every step of the metastasis process can be assumed to be a potential therapeutic target for tailoring therapy (Figure 6), although this may be extremely expensive and may result in different side effects. *In vitro* and *in* vivo models, fundamental in the drug discovery process, do not reflect faithfully the clinical disease due to the complexity and the involvement of so many multiplayers.

Although treatments are available for targeted therapy, the challenge is to design an anti-metastatic drug, with a safe toxicity profile and an improved therapeutic index. To achieve this objective, it is necessary to find a target that is selectively involved in the process of formation of metastasis, but not in other physiological processes.

1.5 Treatment for breast cancer and metastatic breast cancer

To date, over 25 drugs for the treatment of breast cancer and its metastatic form have been approved by the US Food and Drug Administration (FDA). As shown in Figure 7, they may be clustered into three major categories; the 32% of targeted therapy is equally composed of HER-2-targeting therapies and micro-environment-specific targeting molecule.[41]

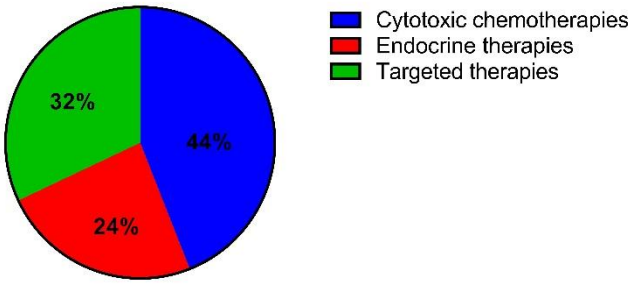


Figure 7. Percentage of metastatic breast cancer treatments approved by FDA, clustered in categories.

Worldwide, the oncology drugs industry is driven by the different therapeutic options such as chemotherapy, targeted therapy, immunotherapy and hormonal therapy. Indeed, the market for anticancer drugs went from $71 billion in 2008, to $91 billion in 2013 and it is predicted to reach $112 billion in 2020.[42]

1.5.1 Endocrine therapy

Patients affected by hormone-receptor positive or metastatic breast cancer are treated with endocrine therapy, as primary systemic therapy, with the intent to achieve palliation of symptoms and improved life quality. Moreover, there is a body of evidence that for ER+ breast cancers, the risk of relapse is reduced by 41% and the mortality associated risk by 31% due to the use of a selective oestrogen receptor modulator.[43]

Unfortunately, the side effects associated with endocrine therapy are various and harmful, such as arthralgia and osteoporosis.[44] In the next section, the most important hormonal therapeutics will be briefly presented.

1.5.1.1 Anti-oestrogens

Tamoxifen (Nolvadex, AstraZeneca, **1**) is a Selective Estrogen Receptor Modulator (SERM) and has the structure represented in Figure 8. It is a pro-drug and has a low affinity for the oestrogen receptor, its target protein, until converted to its active hydroxylated metabolite.

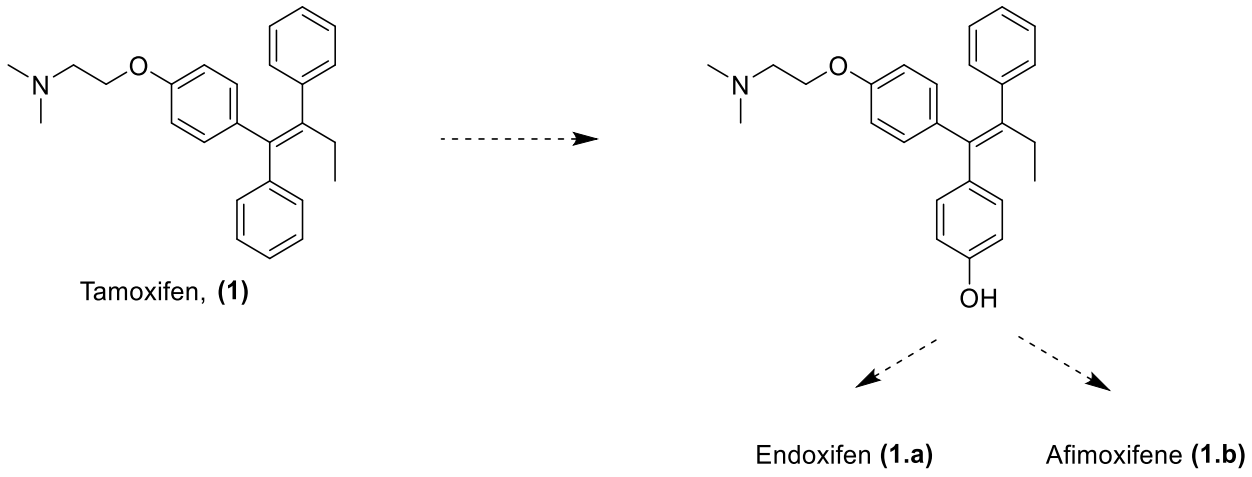
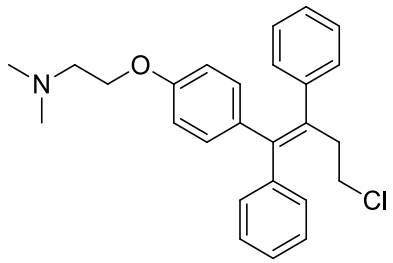


Figure 8. Chemical structure of **1** and its metabolites.

Many actions of this anti-oestrogen drug are mediated by its active metabolites (Figure 8): 4-OH-tamoxifen and endoxifen, produced by the metabolism of tamoxifen by CYP450 in the liver (isoform CYP2D6 and CYP3A4). These active metabolites have around 30-100 times more affinity for the oestrogen receptor than tamoxifen itself. The mechanism of action involves the competitive binding to the oestrogen receptors on tumour cells: the nuclear oestrogen receptor is saturated by tamoxifen. The oestrogen does not bind the receptor and the nuclear complexes of tamoxifen bound to receptor decreases DNA synthesis inhibiting the estrogenic effects, stopping the ER-dependant growth of breast cancer cells.

The effect is cytostatic, rather than cytocidal because the tamoxifen does not cause cell death, but it is useful for preventing cells from dividing. Despite its benefit, it can cause venous thromboembolism and osteoporosis.[45]

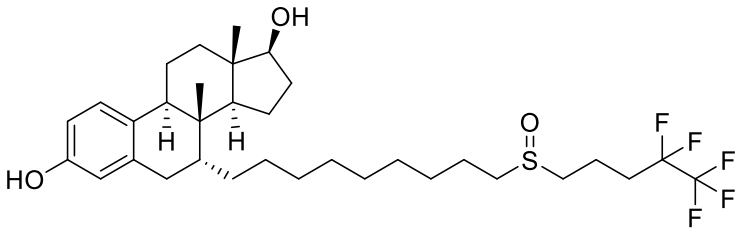
Toremifene (Fareston, Orion Pharma, **2**, Figure 9) is another SERM; it is cross-resistant to tamoxifen and, for this reason, it has not been widely used in the clinic. It is another option compared to tamoxifen in patients named as “poor metabolisers” because it is not metabolised by CYP 2D6.[46]



Toremifene, **(2)**

Figure 9. Chemical structure of **2**.

Fulvestrant (Faslodex, AstraZeneca, **3**, Figure 10) is an oestrogen receptor-antagonist (anti-ER) and a selective ER Down Regulator (SERD) that accelerates the proteasomal degradation of ERs and decreases the number of PRs in breast cancer cells, used for the treatment of hormone-receptor-positive metastatic breast cancer in post-menopausal women who exhibit disease progression after anti-oestrogen therapy. It is administered as a once-monthly injection. Its use in oncological therapy is limited by its expensive price.[46]



Fulvestrant, **(3)**

Figure 10. Chemical structure of **3**.

1.5.1.2 Aromatase inhibitors

Aromatase inhibitors are used for the treatment of ER+ breast cancer. In young women, the majority of oestrogens are produced in the ovaries; in post-menopausal women they are produced thanks to the activity of the aromatase enzyme expressed in adipose, muscle and bone tissues. These inhibitors thus decrease the oestrogen levels by inhibiting the aromatase enzyme activity and show advantages in the treatment of metastatic breast cancer.

Aromatase inhibitors are a first-line treatment of metastatic breast cancer in post-menopausal women. Combination of aromatase inhibitors with an ErbB2 targeted therapy have shown some clinical benefits and are currently studied.[43]

The structures of the most common aromatase inhibitors are presented in Figure 11.

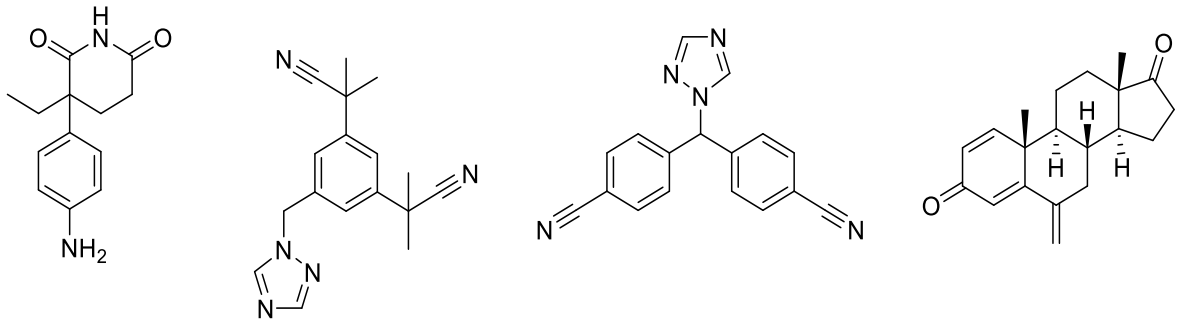


Figure 11. Chemical structure of compounds **4**-**7**.

Aminoglutethimide (Cytadren, Novartis, **4**) is a first generation aromatase inhibitor, initially used as anti-paroxysm. The mechanism of action involves the blockade of oestrogen and steroid hormone synthesis through the inhibition of aromatase enzyme and CYP-450. It is widely used in tamoxifen – resistant patients.

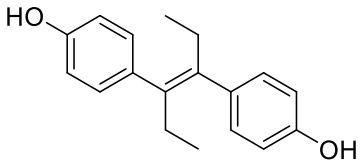
Anastrazole (Arimidex, AstraZeneca, **5**) is a non-steroidal inhibitor of aromatase characterised by a clinical benefit rate equivalent to the one exhibited by tamoxifen, but it has a different toxicity profile.[47]

Letrozole (Femara, INN, off-label uses, **6**) is more effective than tamoxifen and it shows also an improved overall survival rate.[43]

Exemestane (Aromasin, Pfizer, **7**) is used in patients who have progressed on tamoxifen. A phase III clinical trial in 2011 demonstrated that the incidence of breast cancer decreased in postmenopausal women at risk for breast cancer, after exemestane administration.[43]

1.5.1.3 Oestrogens

Diethylstilboestrol (**8**, Figure 12) is a synthetic, non-steroidal oestrogen (structure in Figure 12), which when administered in high dose resulted in a 31% response rate in patients who failed the above endocrine treatments, as shown in a phase II study.[48]



Diethylstilbestrol, **(8)**

Figure 12. Chemical structure of **8**.

Hence, oestrogens themselves are important in the treatment of metastatic breast cancer.

1.5.2 ErbB targeted therapies

Various efforts have been made to develop therapies targeting the ErbB family: monoclonal antibodies directed against the receptor, synthetic tyrosine kinase inhibitors, conjugates of toxins to anti-ErbB antibodies, and ErbB ligands and antisense therapy to Erb receptor. All of these therapeutic modalities have a few advantages such as target selectivity and accumulation of the drug at the tumour site, but also some disadvantages, such as the cytotoxicity and the high cost.
Several therapeutic agents were developed for the Her-2 receptor (mainly directed against the Her2 extracellular domain), because of its pivotal role in breast cancer progression and metastasis.[42]

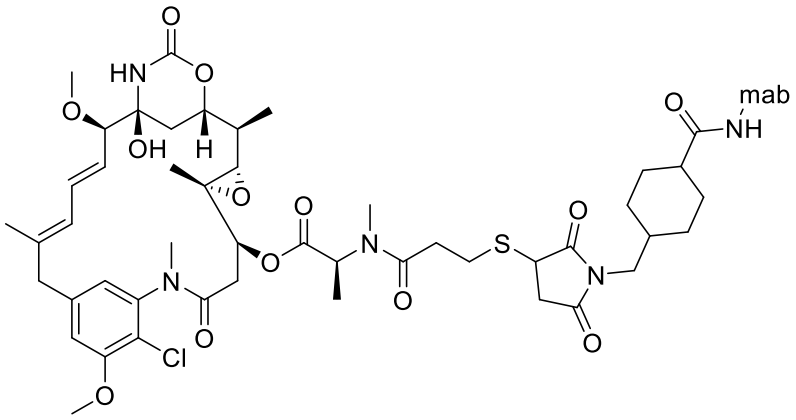
1.5.2.1 Anti Her-2 therapies

Trastuzumab (Herclone, Roche, 9, Figure 13) is a humanised monoclonal antibody interfering with ErbB2 receptor. Its target is the extracellular domain of the Her-2 cell surface receptor in order to both prevent the heterodimer downstream signalling and to activate proliferative MAPK pathways. Hence, it strongly inhibits proliferation in Her-2 over-expression driven breast cancer. Moreover, it promotes accelerated ErbB2 internalisation and degradation, inhibiting the growth of tumour and eradicating established tumour.
It was generally well tolerated in a first clinical trial, although cardiac dysfunction was observed in few cases. Optimal responses during trials in phase II and III led to regulatory approval in the United States of America, as a single agent for the treatment of patients affected by metastatic breast cancer.[46]
It may be used in combination with other chemotherapeutics such as vinorelbine or taxanes such as paclitaxel or docetaxel, and carboplatin: in all these combinations, trastuzumab proved to be clinically safe and showed improved responses. Combination of trastuzumab with anthracyclines has to be avoided because of excessive cardiac toxicity.

Combinations of trastuzumab with other agents, e.g. with newer antiErbB2 agents (such as neratinib or HSP-90 inhibitors) are currently under investigation.[49]

However, patients may exhibit innate and acquired resistance to trastuzumab and some do not show any response at all to this treatment. The mechanisms behind this resistance are still under investigation, but they might involve the loss of PTEN, altered PI3-K components or IGF-IR mediated activation of PI3K/Akt making cells initially sensitive to trastuzumab but later becoming resistant to the treatment.

Trastuzumab emtansine (INN, T-MD, Kadcyla) is an antibody-drug conjugate (Figure 13). This cancer-activated prodrug consists of two parts: the first is the monoclonal humanised antibody trastuzumab (see above), while the second is emtansine (DM1), a cytotoxic and antimitotic maytansine derivative. DM1 binds microtubules in a similar manner to the vinca-alkaloids in blocking mitosis.[54]



T-MD, **(9)**

Figure 13. Chemical structure of **9**.

As single agent, emtansine exhibits a high toxicity profile, but thanks to its conjugation with the monoclonal humanised antibody, it is possible to deliver the cytotoxic agent to target cells with enhanced selectivity.

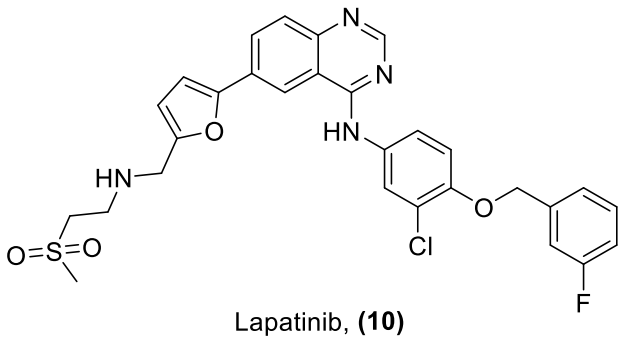
Although is a new option against breast cancer cells resistant to trastuzumab and it has been demonstrated that this association may enhance the effectiveness of previously reported chemotherapy, it is expensive and the National Institute Health and Care Excellence (NICE) did not approve it for use in the United Kingdom.[55]

1.5.2.2 Inhibitors of tyrosine-kinase activity

Tyrphostins are inhibitors of tyrosine-kinase activity that suppress the tyrosine kinase activity of ErbB2-positive breast cancer cells. Several strategies are currently under development, such as recombinant immune-toxins directed against HER. Additionally, down-regulation of Her-2 expression could be effected by Her-2 anti-sense oligonucleotides, which exhibit an enhanced growth inhibitory and pro-apoptotic activity.

Emodin is a non-selective Her-2 inhibitor, isolated from the root of *Rheum Palmatum L*. It was carefully studied because of its anti-metastatic effects.[13] Several studies show that emodin significantly down-regulates NF-κB DNA-binding activity.[51] It is a tumour growth inhibitor.

Another therapeutic belonging to the inhibitor of tyrosine-kinase is lapatinib (Tyverb, Glaxo Smith Kline, **10**), depicted in Figure 14.



Lapatinib, **(10)**

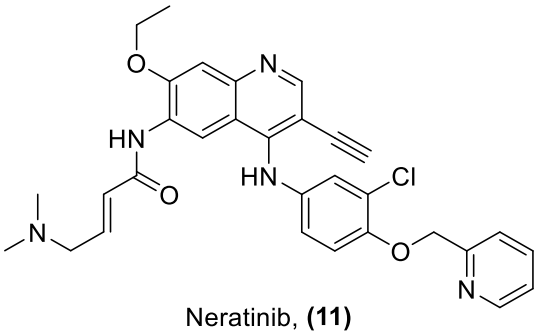
Figure 14. Chemical structure of **10**.

It is a small orally active and dual tyrosine kinase inhibitor. Its action results in interruption of the Her2/neu and Epidermal Growth Factor Receptor pathways. It is widely used in patients affected by triple negative breast cancer or in patients who have developed metastasis that have progressed after previous treatment with trastuzumab or taxane derived drugs. It is also used in trastuzumab-resistant tumours. It may cross the blood-brain barrier and reduces the presence of metastasis in the central nervous system.

The combination of lapatinib with the antimetabolite capecitabine shows a 51% reduction in the risk of disease progression in HER2-positive breast cancer patients. It is well-tolerated, even if the most common side effects are nausea, rashes or cardiac dysfunction.

Neratinib (HKI-272, Puma Biotechnology, **11**, Figure 15) is an orally administered, dual inhibitor of both ErbB2 and epidermal growth factor receptor kinase (EGFR), acting on the proliferation of

EGFR-dependent cells. It is an irreversible binding inhibitor; it inhibits the cell cycle regulatory pathways and thus decreases cell proliferation.

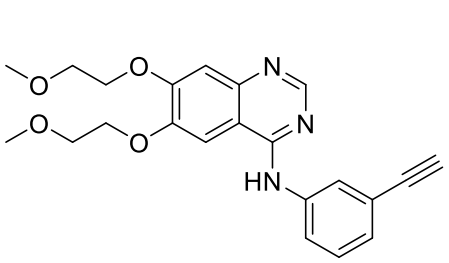


Neratinib, **(11)**

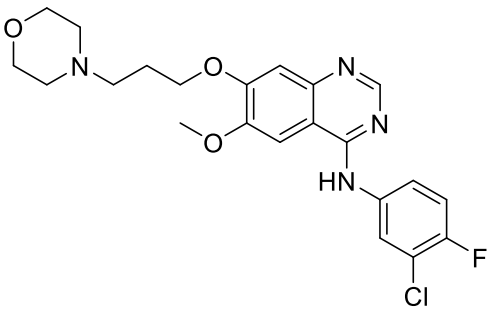
Figure 15. Chemical structure of **11**.

The main side effect is diarrhoea in about 30% of patients. The anticancer activity of neratinib seems promising not only in combination with paclitaxel or capecitabine, but also as single agent.[51] It is actually in phase III trials, encompassing three different studies: as single agent in early stage breast cancer patients who were pre-treated with trastuzumab; a study of neratinib versus lapatinib and capecitabine in metastatic breast cancer patients who were pre-treated with trastuzumab; and finally, as combination therapy with paclitaxel versus trastuzumab + paclitaxel in patients with metastatic breast cancer.

Erlotinib (Tarceva, Genentech, **12**, Figure 16) is a reversible Her-1 inhibitor, which suppresses Her-2 transactivation. It has been approved for the treatment of pancreatic cancer, metastatic non-small cell lung cancer or unresectable breast cancer, as a single agent.[58]



Erlotinib, (**12**)



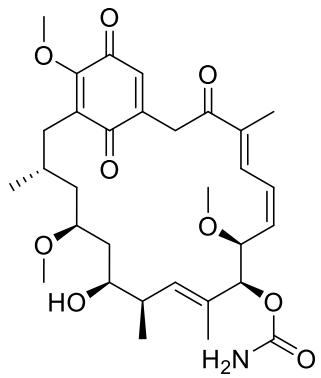
Gefitinib, (**13**)

Figure 16. Chemical structure of **12**, **13**.

Gefitinib (Iressa, Astra Zeneca, **13**, Figure 16) is an inhibitor of Her-2/Her-3 hetero-dimers. It has been approved in U.S.A. and Europe for the treatment of metastatic non-small cell lung cancer in patients unsuccessfully treated with platinum-based and docetaxel treatments.[53] In metastatic breast cancer, it only showed significant clinical benefits when in combination with other chemotherapeutic agents.[54]

1.5.2.3 HSP-90 Inhibitor

17-AAG (Tanespimycin, Bristol-Myers Squibb, **14**, Figure 17) is a heat shock protein 90 (Hsp-90) inhibitor. Hsp-90 is over-expressed in human cancer cells and is fundamental to stabilise several proteins required for the survival of cancer cells. It acts as a molecular switch to regulate and restrain the kinase activity of Her-2 receptor and its ability to form active hetero-dimers.[56] It has a good clinical safety profile in combination with trastuzumab and is well tolerated. It inhibits tumour growth showing interesting anticancer activity.[49]



Tanespimycin, **(14)**

Figure 17. Chemical structure of **14**.

1.5.3 Angiogenesis inhibitors

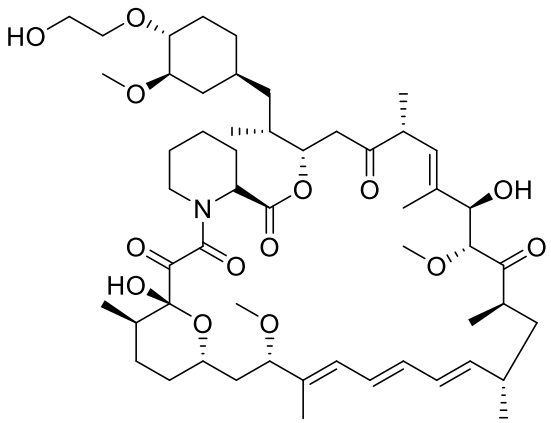
Bevacizumab (Avastin, Genentech/Roche) is a recombinant humanised monoclonal antibody. It blocks angiogenesis by inhibition of the Vascular Endothelial Growth Factor A (VEGF-A) protein. Its use in combination with paclitaxel was approved by the European Commission in 2007, as first-line treatment of metastatic breast cancer. One year later, the Food and Drug Administration (FDA) also approved it for the treatment of metastatic breast cancer. However, in 2010, the FDA withdrew approval as a therapeutic option for metastatic breast cancer because of data from different clinical

trials, where bevacizumab failed to slow disease progression.[57] The observed poor activity as a single agent has been improved in combination study with paclitaxel, although there are concerns about severe side effects such as bleeding, hypertension, thrombosis.

1.5.3.1 mTOR inhibitor

The mechanistic target of rapamycin (mTOR) plays a leading role in angiogenesis in several solid cancers, such as breast cancer.[58]

Everolimus (trade name Certican, Novartis, **15**, Figure 18) is an mTOR inhibitor, used in post-menopausal women with advanced hormone-receptor-positive, HER2-negative type cancer, in conjunction with exemestane. It was approved by the FDA in 2012. It affects tumour cells growth and proliferation.[59]



Everolimus, **(15)**

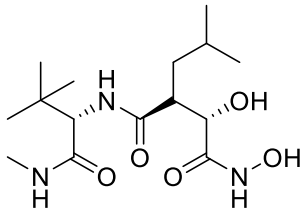
Figure 18. Chemical structure of **15**.

1.5.4 MMP inhibitors

Owing to their importance in the development of cancer metastasis, several efforts focused on design of matrix metalloproteinase inhibitors (MMPIs) as new therapeutic agents in several cancers.

Unfortunately, the majority of these inhibitors used in late stage malignancies showed a poor beneficial effect. Their activity is often associated with adverse reactions and side effects such as muscle and bone pain.

Marimastat (British Biotech, **16**, Figure 19), a peptidomimetic MMP inhibitor, was used in the treatment of breast cancer and small cell lung cancer and non-small cell lung cancer as an anti-neoplastic drug: due to the poor performance in clinical trials phase III, its use was discontinued.[60]



Marimastat, **(16)**

Figure 19. Chemical structure of **16**.

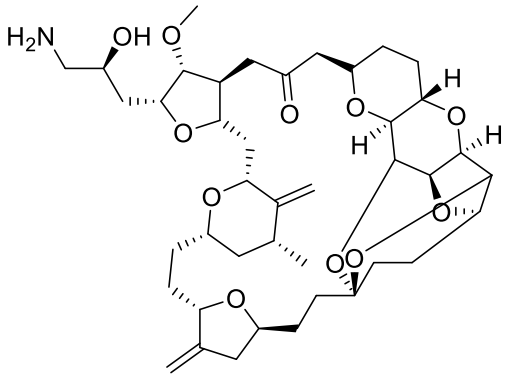
1.5.5 Taxane

Nanoparticle albumin-bound paclitaxel ([nab-paclitaxel] or ABRAXANE® ABI-007, American Pharmaceutical Partners, Inc. / American Bioscience, Inc) is a new generation formulation of paclitaxel. Nab-paclitaxel is characterised by a higher tolerability profile than the solvent-bound paclitaxel.[61] It is an anti-microtubule agent. It disrupts the microtubular network and inhibits cell division: a mechanism known as "frozen mitosis." Moreover, nab-paclitaxel also is able to increase paclitaxel delivery to the tumour cells. Nab-technology utilises albumin to transport paclitaxel directly to the cancer cells via receptor-mediated transport mechanism.[62]

1.5.6 NON-TAXANE

Eribulin mesylate (trade name Halaven, Eisai Co, **17**, Figure 20) is a non-taxane, microtubule inhibitor used in the cytotoxic chemotherapy of patients with metastatic breast cancer who have already been treated unsuccessfully with different chemotherapeutic treatments (such as anthracycline and taxane).[63]

It is an analogue of Halichondrin B, a marine sponge product. It inhibits microtubule growth and it sequestrates tubulin into aggregates.



Eribulin, **(17)**

Figure 20. Chemical structure of **17**.

The most commons side effects, observed over a period of 9 years, are: drop in white blood cells, anaemia, hair thinning. It is currently in Phase III clinical trials. Its use in other type of cancer was approved in 2010.[61]

In conclusion, despite the improvements in breast cancer detection and the therapeutic options nowadays available, the 5-year survival rate of women with primary breast cancer has been improved, but there has not been any real progress in the effective treatment of metastatic breast cancer patients. Indeed, although different combinations as therapeutic approaches are currently under investigation and a more tailored approach to therapy is in development, the 5-year survival rate percentage of patients with metastatic breast cancer still remains low and distant reoccurrence is still common and basically incurable. Because 90% of cancer patient deaths are due to metastasis, it is well worth focusing attention on the development of anti-metastatic small drug-like molecules. These molecules should target a particular and molecular player with high specificity to reduce or even prevent side effects, improve treatment efficacy and lower overall treatment costs. Indeed, one of the major disadvantages of targeting cell division in cancer is linked to the targeting of complex pathways machinery and multiplayers in cell signalling and general toxicology. Therefore, there is a pressing need for the identification of novel molecular targets, which are exclusively involved in the metastatic cascade of the disease, and then for the development of drugs acting on these targets.

1.6.1 Roles of NF-ΚB

NF-κB is the Nuclear Factor that binds the Kappa-light-chain-enhancer of activated B cells (NF-κB). Since its discovery in 1986, NF-κB protein family has increasingly been recognised as a crucial player in inflammation, innate immunity and in many steps of cancer initiation and progression.[71] It is an essential "rapid acting" transcription factor, expressed in all cell lines and involved in the regulation of several target genes with different functions, as depicted in Figure 21.[66]

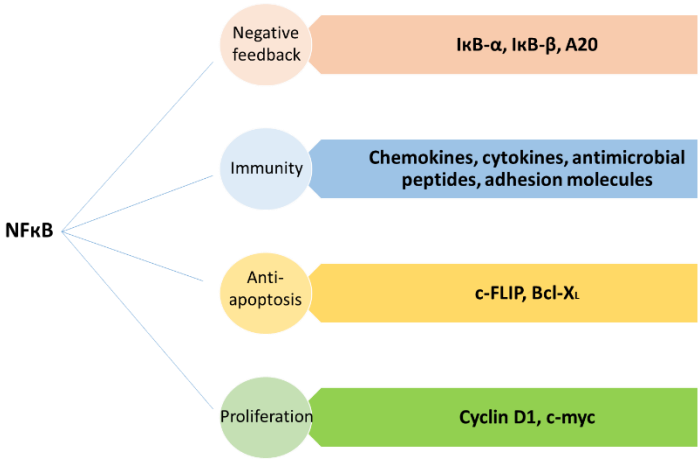


Figure 21. NF-κB contributes to the induction of four classes of genes.

As an early response gene, it may modulate both immune and inflammatory cellular responses, cellular proliferation and apoptosis in response to a plethora of stimuli.[67]

NF-κB protein family is involved also in cytokine production, synaptic plasticity, memory, cell migration and cell survival.[65]

A variety of stimuli may activate NF-κB pathway: pro-inflammatory cytokines, Tumour Necrosis Factor (TNF) or Interleukin-1 (IL-1), growth factors, lymphokines, oxidant-free radicals, inhaled particles, viral infection, UV irradiation, B or T-Cell activation (Figure 21).[68] Because of its response to several stimuli, it is not surprising that NF-κB pathway is constitutively activated in different diseases (such as hepatitis, sepsis, neurodegeneration, asthma) and in many types of cancers (breast, lung or colon cancer as well as in haematological malignancies).[69]

1.6.2 NF-KB: role in tumourigenesis

It is well established that there are seven leading alterations to normal cell physiology in the development of tumourigenesis: self-sufficiency in growth signals; insensitivity to growth inhibition; evasion of apoptosis; immortalization; sustained angiogenesis; tissue invasion; metastasis.[70]

NF-κB may induce all of these seven cancer hallmarks. NF-κB overthrows the balance between apoptosis and cell proliferation, promoting cell proliferation and suppressing programmed cell death.[71]

Hence, NF-κB may inhibit apoptosis in two ways: it may induce the expression of multiple anti-apoptotic proteins (such as cFLIP); or, it may interfere with the expression of pro-apoptotic proteins (death receptors 4 and 5) and their activity.[68]

NF-κB plays an important role in cancer invasion and angiogenesis. NF-κB regulates expression of several leading factors in cancer invasion and angiogenesis, such as matrix metalloproteinase 2 and 9, serine protease urokinase-type plasminogen activator, intracellular adhesion molecule 1, vascular cell-adhesion molecule 1.[66] Additionally, NF-κB activation regulates also production of angiogenic factors (chemokines, growth factors).[67]

Moreover, NF-κB promotes cell cycle progression: it regulates the expression of genes involved in the cell cycle machinery (cyclin D1, D2, D3, c-myc) (Figure 21).[72]

NF-κB interacts and cooperates with different signalling molecules and many pathways.[73] Important node of crosstalk is mediated by transcription factors, such as STAT-3.

Recently, a liaison between NF-κB and STAT-3 signalling pathway has been reported: they are persistently activated in many cancers with poor prognosis, such as breast, lung and prostate cancers.[74] Moreover, NFκB controls STAT-3 signalling pathway activation, by inducing the expression of particular chemokines.[75, 76] Mutations and deregulations occur in upstream receptors or in genes involved in cell proliferation, survival, angiogenesis and tissue repair in response to autocrine and paracrine factors produced within the tumour microenvironment.[77] Several chronic inflammatory conditions predispose to cancer: evidence from inflammatory microenvironment of the neoplastic tissues confirmed the idea that the inflammation may be considered a cofactor in oncogenesis for different types of cancer.[73]

Another node of co-operativity is mediated by the proto-oncogene Her-2. Although normal endogenous levels of Her-2 and NF-κB are essential for the physiological development of mammary gland, their amplification is responsible for the most aggressive form of metastatic breast cancer.[76] As a result, in ER- tumours, a link between Her-2 over-expression and NF-κB activation has been

found. Moreover, cancer cells characterised by activation of NF-κB and HER-2 are more resistant to the usual cancer therapy and radiotherapy.[67]

Additionally, a correlation between Her-2 over-expression and STAT-3 activation has been reported.[79] It has been proposed that the link between STAT-3 and NF-κB is represented by the proto-oncogene Bcl-3. The exact mechanism has still to be elucidated, although the relationship has been proved in *in vivo* models.[80]

Due to the pivotal and different roles that NF-κB plays, it has been strongly suggested that NF-κB signalling pathway is an interesting target for cancer prevention and treatment, in human malignancies, which have an NF-kB dependency.[68, 72] Attempts to target NF-κB genes have been investigated far-back. At the same time, due to its involvement in different functions, targeting directly NF-κB to design specific inhibitors may be detrimental because of its involvement in the innate immunity. Thus, also targeting STAT-3 may be noxious for the normal tissue homoeostasis.

In this scenario, Bcl-3 emerges as a more promising molecular target candidate to design small drug-like molecule as a novel therapeutic option for metastatic breast cancer patients for several reasons: it is a downstream cofactor in both NF-κB and STAT-3 pathway; it is involved in metastatic progression of breast cancer, but without unfavourable effects on normal mammary gland function or other physiological functions.[80] It is a more selective target involved in cell migration and pro-metastatic process. Stronger evidence to support Bcl-3 as a novel and promising target will be provided in section 1.7.

1.6.3 NF-κB pathway

The NF-κB protein family is made up of two subfamilies: "Rel proteins" and "NF-κB proteins". The former consists of three members: c-Rel, Rel B and Rel A (p65). while "NF-κB proteins" consists of p50/p105 (NF-κB1) and p52/p100 (NF-κB2): in contrast with Rel members, they are synthesised as pro-forms (p-105, p-100) and are proteolytically processed to p-50 and p-52, respectively.

All the members of the NF-κB family share a highly conserved DNA-binding/dimerisation domain, named Rel Homology Domain (RHD): it consists of 300 amino acids and it is located at the *N*-terminus. The RHD controls different functions[81]: 1) DNA binding (DNA binding domain or DBD); 2) dimerisation; 3) cellular localisation of the monomeric components of this protein family via the Nuclear Localisation Sequence, (NLS); 4) the double interaction between inhibitory factors named IκBs and each of the five members of the NF-κB family.[82]

The members of NF-κB protein family are distinguished by their long C-terminal domains: the Rel proteins exhibit the transcriptional activation domains (TDs), responsible for the positively regulation of Rel proteins by IκB binding.[83]

Conversely, C-terminal ends of NF-κB proteins do not have the TDs, but seven copies of ankyrin repeats, as depicted in Figure 22.[84] Lacking the transactivation domain, NF-κB proteins are transcriptionally inactive. Upon binding with a member containing a transactivation domain (such as Rel B o Bcl-3), NF-κB heterodimers are activated.[81]

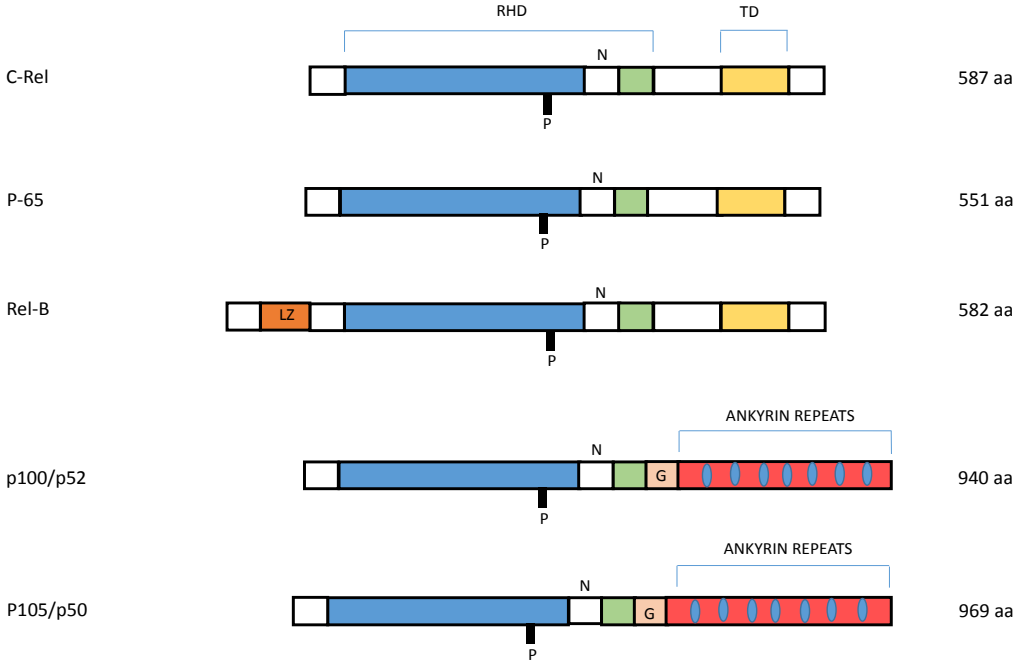


Figure 22. Domain organisation of NF-κB protein family members. RHD = Rel Homology Domain; TD = Transcriptional Activation Domain; LZ = Leucine zipper; N = NLS; P = PKA Phosphorylation motif; G = Glycin rich region [34]. The number of amino acids per each protein is indicated on the right-hand side.

Nearby the RHD, only in the NF-κB1 and NF-κB2 proteins, there is a glycine-rich region (GRR) which acts as a processing signal for generation of p50 from p105 in unstimulated cells (and p-52 form p-100). GRR is not the site of processing: the cleavage occurs at a specific distance downstream of the GRR and involves an ATP-Mg^{2+}-dependent protease. In contrast, in stimulated cells, p105 is phosphorylated at the C-terminus and undergoes ubiquitination and degradation via the proteasome and it is translocated in the nucleus.[83]

NF-kB protein family shows three possible functioning ways: firstly, it is able to alter kB-site specificity as part of a heterodimer with TD-containing family members; secondly, NF-kB may repress transcription as part of a homodimer when bound to kB sites; thirdly, NF-kB may promote transcription through recruitment of other TD-containing proteins to kB sites.[84]

All the five members associate with one another to form transcriptionally active homo- or hetero-dimeric complexes. Rel B is the only exception since it is able to interact only with p50/p52.

Classically, heterodimers of NF-κB protein family members are retained inactive in the cytoplasm by interactions with inhibitory cytoplasmatic IκB proteins (IκB). Upon inductive stimuli mediated activation, phosphorylation and degradation of IκB kinase allow the translocation of NF-κB heterodimers in the nucleus where they regulate gene expression by binding to DNA and to other co-activators.[91]

Thus, NF-κB activity is tightly regulated by binding with IκB proteins. Structurally, the IκB protein family is characterised by multiple copies of ankyrin repeats: IκBs retain NF-κB protein family members inactive in the cytoplasm masking their NLS with the ankyrin repeats. IκBs are classified in three groups: the first consists of three IκB proteins, named as IκBα, IκBβ, IκBε.[84, 85] The second group comprises p100 and p105, because of their structural similarity in the ankyrin repeats motif, with the other IκB members. The third group includes IκB proteins (IκBς, $IκB_{NS}$ and Bcl-3), atypical members because of their nuclear localisation.[86]

A lot of research and scientific efforts focus on Bcl-3, mainly because of the presence at its C-terminus of a transactivation domain, essential for transcriptional activity. The atypical co-activators IκBs (Bcl-3 and I κBς) may either inhibit or promote the transcriptional responses to NF- κB.[89] They interact with complexes containing p50 and/or p-52 and they are able to promote transcription of a subset of NF-κB responsive genes.[90]

The inhibitory cytoplasmatic IκB proteins have a different affinity for each NF-κB dimers, exhibiting differences in their specific tissue expression. They are regulated by proteolysis and phosphorylation by the IκB kinase (IKK). IKK consists of a heterodimer of three elements: two catalytic subunits (IKKα, IKKβ) and a "master" regulatory protein subunit named NEMO or IκBγ (involving the activation of genes implicated in inflammation, immunity, cell survival, and other pathways).[88]

There are several levels at which NF-κB activity is tightly regulated.[20] The IκB proteins and the IκB kinase complex (IKK) responsible for its phosphorylation are the primary mechanisms of regulation of NF-κB. The second mechanism of regulation involves post-translational modifications of IκB and IKK. IκB, IKK and A20 are regulators of the NF-κB proteins, but at the same time they are NF-κB dependent: they generate auto-regulatory feedback loops in the NF-κB response.[87]

It is well established that there are three pathways leading to the activation of NF-κB in response to appropriate stimuli: pathway 1 or canonical, pathway 2 or non-canonical and pathway 3 or atypical as shown in Figure 23, 24, 25.[85]

1.6.3.1 Canonical pathway

Various signals trigger the canonical pathway: they are mostly physiological and mediated by innate and adaptive immune receptor or cytokine receptors, such as TNF, IL-1, IL-1R, antigen receptors and pattern-recognition receptors, Toll-like receptor 4.[85]

The canonical pathway (Figure 23) depends on IKKβ and NF-κβ essential modulator named NEMO.[92]

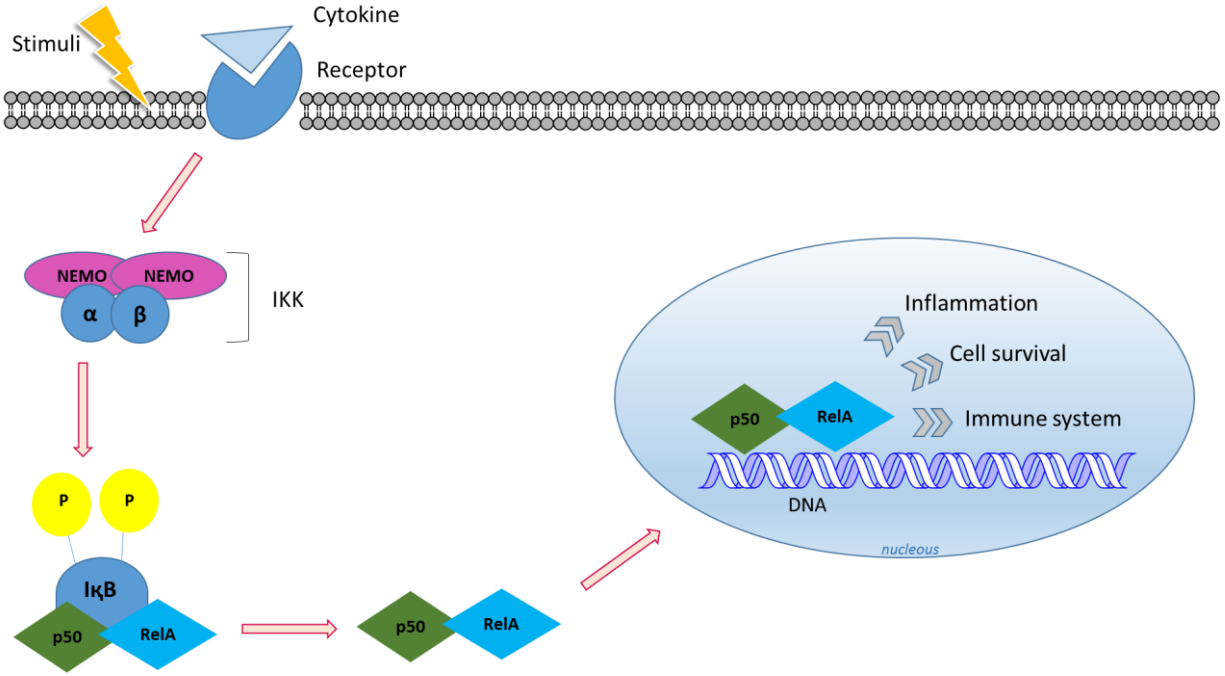


Figure 23. The canonical NF-κB signal transduction pathway.

IKK complex is activated by NEMO through IKK-mediated IκBα phosphorylation of two serine residues located in the IκB regulatory domain, ubiquitination and subsequent degradation via the proteasome, resulting in rapid and transient nuclear translocation of the prototypical NF-κB heterodimer RelA/p50.

The dissociation of IκB from NF-κB dimers allows to NF-κB to enter the nucleus and switch on specific genes, which show DNA-binding sites for NF- κB nearby. When the genes are activated, a physiological response occurs: this can be either an inflammatory or immune response as well as a cell survival response or cellular proliferation.

Moreover, NF-κB is able to promote the expression of its repressor (IκBα): after its synthesis, NF-κB is switched off thanks to the formation of an auto-feedback loop which involves the exhibition of oscillating levels of NF-κB.[93]

1.6.3.2 Non-canonical pathway

The non-canonical pathway, in Figure 24, is activated by different stimuli, such as lymphotoxin-α, BAFF, RANKL, CD40 ligand, lymphotoxin-β2.

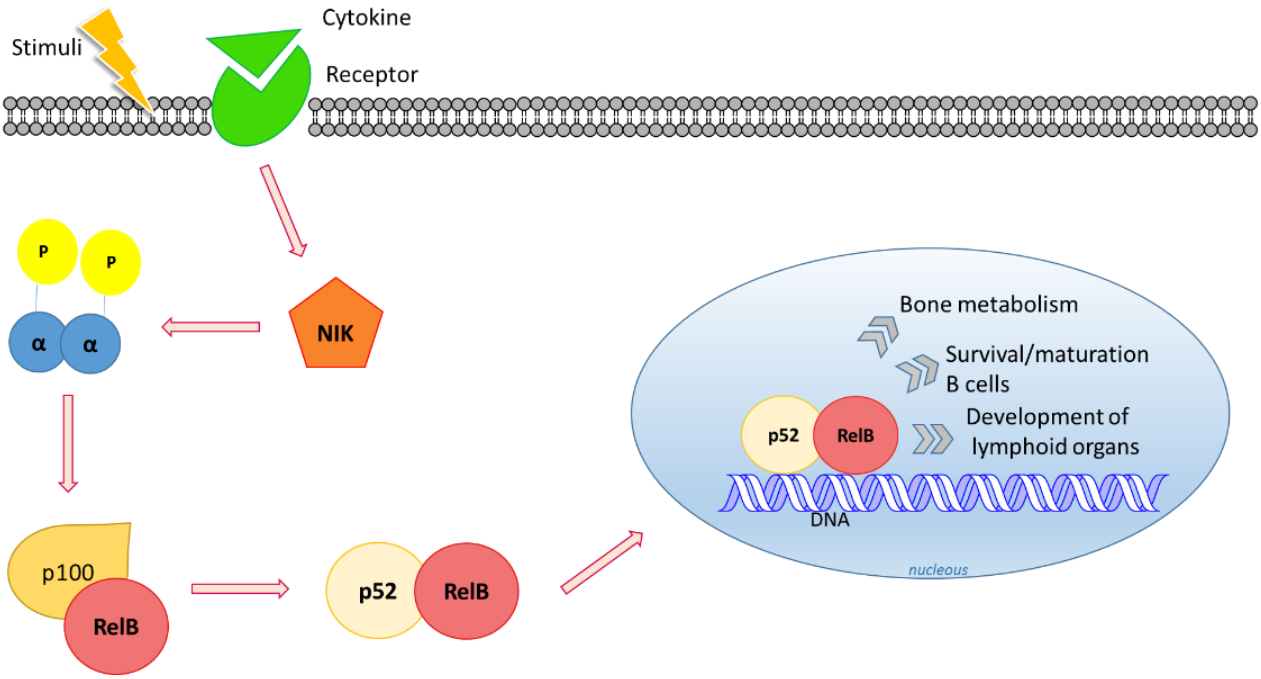


Figure 24. The non-canonical NF-κB signal transduction pathway.

The non-canonical pathway depends on IKKα-mediated phosphorylation of p100 associated with RelB and on NF-κB inducing kinase, named NIK. The non-canonical pathway leads to partial processing of p100 and generation of p52- RelB complexes.[94] Dimers of p52 are able to move into the nucleus not following degradation and ubiquitination by the proteasome, but thanks to the NF-κB inducing kinase. NIK is able to phosphorylate two serine residues adjacent to the ankyrin repeat C-terminal IκB domain of p-100, leading to its partial proteolysis and liberation of the p-52/Rel-B complex. NF-κB2 precursor protein p-100 is processed into a mature p-52 subunit. Hence, p-52 may form dimers with Rel B and regulate a distinct class of genes. The canonical and non-canonical pathway seem to be independent of each other. However, recent studies show that the RelB and p-52 synthesis is controlled by canonical pathway, due to the action of IKK2-IκB-RelA: p-50 signalling.[95]

1.6.3.3 Pathway 3

Bcl-3 is usually expressed in low level in unstimulated cells, but it may be induced by NF-kB pathway activation.[87]

NF-κB is aberrantly activated in solid tumours and it modulates Bcl-3 expression: activation of NF-κB signalling pathway leads to overproduction of Bcl-3, as reported in several malignancies.[86] The signalling cascade involving Bcl-3 has not been completely elucidated. It is established that it is up-regulated by different cytokines such as TNF-α, IL-A, IL-1, IL-6, IL-10, IL-12 and adiponectin.[87] As depicted in Figure 25, these cytokines may activate IKK to induce processing of p-105 precursor to p-50; p-50 forms dimers and translocates into the nucleus.[88]

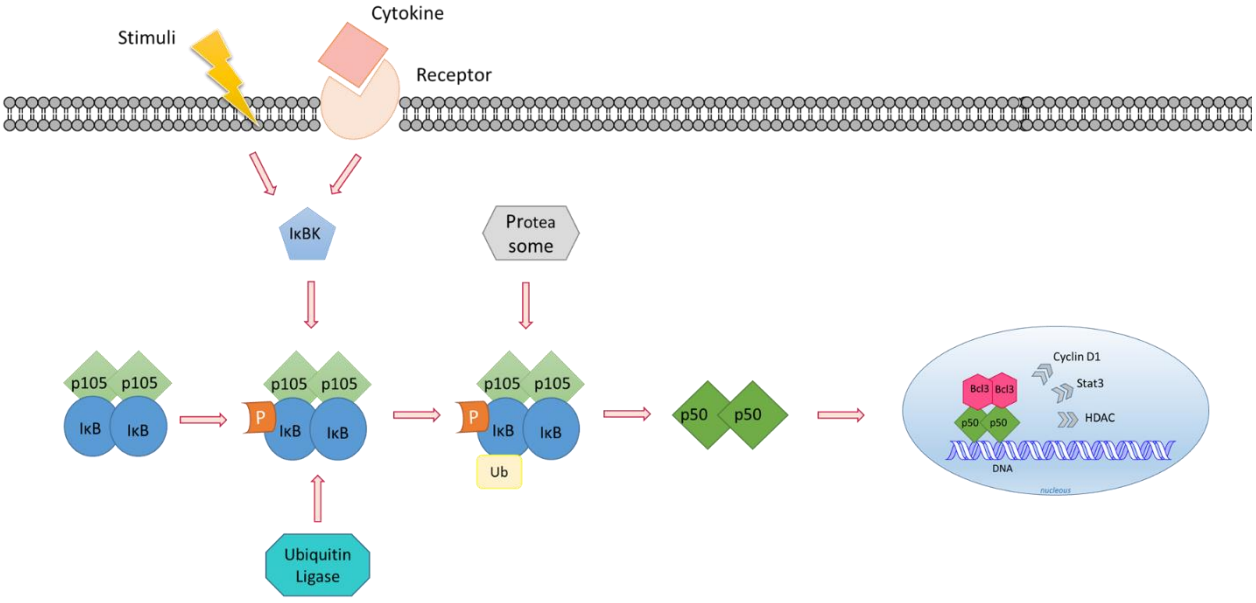


Figure 25. Bcl-3 signalling cascade.

Over-expression of Bcl-3 causes p50 to be released from cytosolic p105-p50 complexes and to translocate into the nucleus, increasing its nuclear localisation.[90]

In presence of the NF-κB homo-dimer $(p50)_2$, Bcl-3 forms a ternary complex with DNA and stimulates transcription of a different panel of genes (cyclin D1, SLP1, CXCL-1, lamin-β-2, HDAC-1, HADAC-4, HADAC-6)[68] activating the metastatic progression of breast cancer. The precise mechanism is still not clear and it has to be further investigated.[65]

1.7 Biological rationale for Bcl-3 as promising novel biological target

B-cell lymphoma 3-encoded (Bcl-3) protein is a protein identified about 30 years ago and encoded by Bcl-3 gene in the humans. It is a proto-oncogene, deregulated and over-expressed in haematopoietic and solid tumours, such as breast, colorectal, stomach, urothelial cancers.[95] Bcl-3 in complex with p50 was found to be over-expressed also in nasopharyngeal carcinomas and

hepatocarcinoma.[89] It is involved in the induction of proliferation and inhibition of cell death, but its role in endogenous solid tumours has to be still investigated.[104]

Bcl-3 is an atypical, non-inhibitory member of the IκB protein family, mainly localised in the nucleoplasm. It plays an important role as a transcriptional co-activator, through its association with the NF-κB homodimers (p50 or p52).

As transcriptional co-activator, Bcl-3 may associate with general transcription factors (TFIIB, TATA-binding protein, TFIIA) and other co-activators (CBP/p300, Tip60 histone acetyltransferase).[109]

The Bcl-3 protein contains seven ankyrin repeats as IκB proteins: because of this structural homology, it is really close to this family of proteins. IκBα is the most studied protein of the IκB protein family, and for this reason, it has been used as a comparison to describe Bcl-3 structure (Figure 26).

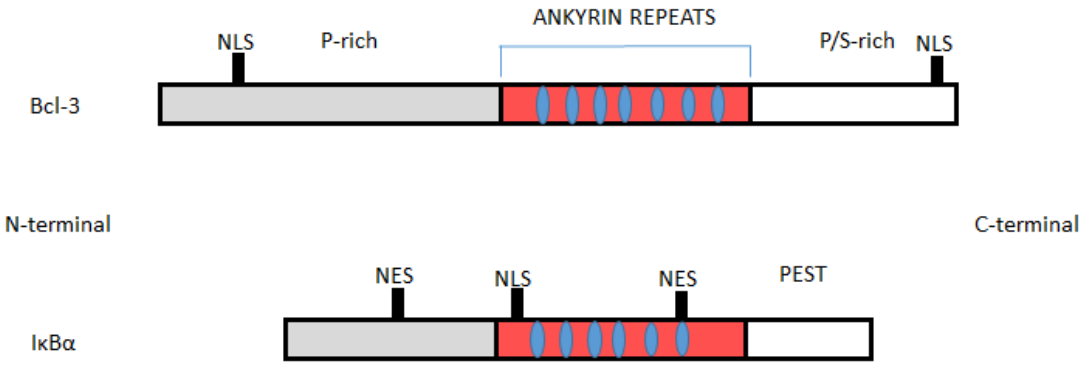


Figure 26. Structure of Bcl-3 and IκBα. NLS=nuclear localisation sequence; NES=nuclear export signal; P=proline; S=serine.

Although the two structures are really similar in the central ankyrin repeats, several differences may be found. Bcl-3 lacks the nuclear export signal and the PEST domain, whereas it has two nuclear localisation sequence. Bcl-3 has seven ankyrin repeats and IκBα has six ANK repeats. The N-terminal portion of Bcl-3 is enriched by proline, the C-terminal region is rich in serine and proline, thus rich in potential phosphorylation sites.[99] Bcl-3 phosphorylation state may modify its activity and function. These differences are important because they may explain the selectivity towards several NF-κB species, the association of Bcl-3 with its NF-κB partner bound to DNA and also the stoichiometry of the Bcl-3:p50 complex.[95]

The ankyrin repeats domains are involved in the protein-protein interactions and may specify interactions with members of IκBs: ANK 1 and 2 recognise the nuclear localisation sequence; ANK 5 and 6 contact the dimerisation domain of p65; the ANK repeats 4 and 6 contact a cluster of hydrophobic residues in p50.[99]

Two different mechanisms of regulation of Bcl-3 have been reported: phosphorylation and ubiquitination.[100, 101]

Bcl-3 is phosphorylated by multiple kinases: for instance, the C-terminal domain of Bcl-3 is phosphorylated by GSK-3β.[83] Phosphorylation of Bcl-3 modulates its activity, attenuates its oncogenicity and triggers Bcl-3 degradation via the proteasome.[100] The expression of few Bcl-3 target genes is affected by GSK-3β-mediated phosphorylation: SLP, Cxcl-1, CYPIBI. Conversely, GSK-3β phosphorylation of Bcl-3 does not affect interaction with p50 or p52.

Another post-translational modification, the polyubiquitination, targets the N-terminal domain of Bcl-3.[101] The CYLD protein deubiquitinates Bcl-3 and inhibits its nuclear translocation.

Little is know about the ubiquitination mechanism and the exact mechanism is still an open question.[102]

Acetylation is another post-translational modification, which may activate Bcl-3 and gene transcription. The acetylation of Bcl-3 mediated by histone deacetyltransferase has to be further investigated as no further details about the precise pathway are known to date.[93]

As mentioned before, Bcl-3 acts as a transcriptional co-activator of NF-κB pathway and it is associated with malignant progression in Her-2-positive breast cancer.[80, 103] Nuclear extracts from breast cancer cell lines show high nuclear levels of Bcl-3 and p50, but variable expression of p52.[104]

A correlation between Bcl-3 expression and an increased cellular proliferation and survival has been established, even if the precise mechanism has not still been elucidated.[98]

Moreover, a liaison between the NF-κB activation and higher expression level of Bcl-3 has been proved in different cancer cell lines.[80]

A link between Bcl-3 and STAT-3 has been reported.[81, 105] In *in vivo* studies using mouse models of Her-2 positive breast tumours it was possible to determine a positive correlation between the Her-2 status, NF-κB activation and Bcl-3 expression level.[80]

The pro-metastatic effect of Bcl-3 was observed in both murine and human breast cancer cell lines in Boyden chamber migration assays, cell cycle assays, cell viability assays.[105]

Briefly, cells with higher Bcl-3 expression level exhibit higher migratory capability compared to cells with lower Bcl-3 expression level. Moreover, Bcl-3 *in vitro* knockdown does not affect cell viability and cyclin D1, in contrast to recent findings showing a correlation in elevated levels of Bcl-3, p50/p52 and cyclin D1 in human breast cancer.[102,106,107] Additionally, *in vitro* Bcl-3 knockdown did not affect NF-κB activity.

Moreover, the pro-metastatic activity of Bcl-3 has been further supported in *in vivo* experiments.

In vivo knockdown studies in Her-2 over-expression driven breast cancer in different mouse models, shown that Bcl-3 deficiency did not affect the primary tumour, but it reduced the occurrence of metastases by 80% without any effects on the normal mammary gland function.[80] It has been demonstrated that Bcl-3 deficiency significantly reduced metastatic tumour burden without any effects on the primary tumour growth. In summary, *in vivo* studies have demonstrated that deletion of Bcl-3 leads to several consequences: decrease in secondary tumour colonisation, decrease in cell motility and mobility, but without any effects on the normal mammary gland function.

As aforementioned, although both NF-κB and STAT-3 may be an attractive target for the treatment of metastatic breast cancer, several disadvantages emerge in the development of a putative inhibitor for those targets.

To sum up, in Her-2 driven breast cancers, it has been demonstrated activation of the NF-κB pathway, leading to over-expression of Bcl-3 and increased nuclear localisation of p-50. Formation of the Bcl-3/p-50 complex activates transcription of genes involved in the metastatic progression of breast cancer. Suppression of Bcl-3 has been demonstrated not to have cytotoxic effects, but to inhibit formation of metastases both *in vitro* and *in vivo* models.

Hence, the proto-oncogene Bcl-3 in complex with p-50 represents an alternative and promising molecular target and approach for different reasons: firstly, selectively involvement in the metastatic cascade of breast cancer acting as transcriptional co-activator; secondly, the non-cytotoxic effect when suppressed, as demonstrated in *in vivo* and *in vitro* metastatic breast cancer models.

In conclusion, the design of inhibitors that selectively disrupt the protein-protein interaction, or in other words, the binding between Bcl-3 and p-50 is a new promising therapeutic option as anti-metastatic drug in metastatic breast cancer.

1.8 Related work

The drug discovery process involves different stages, as schematised in Figure 27. Target identification (TI) is the first step. Successively, target validation (TV) demonstrates the importance of target in the disease state. Hereafter, the hit identification (HI) step aims at identifying new active molecules.[110] Computational methodologies are crucial in this step: virtual high throughput screening (vHTS) is primarily used to screen *in silico* hundreds of thousands of compounds based on their binding affinity for the target protein.[111]

Ten or hundred compounds are selected and their biological evaluation is assessed to select a lead compound in the lead identification (LI) step. Structure-activity relationships drive the synthesis of new analogues to improve the biological activity and enhance the drug-like profile. The lead

optimisation phase (LO) requires the study of the pharmacokinetics property to improve the profile of lead compound, which now is a candidate drug (CD). The development phase may start: clinical trial I, II and III assess a safe and well-tolerated dose and also the efficacy and safety of the new drug.[112]

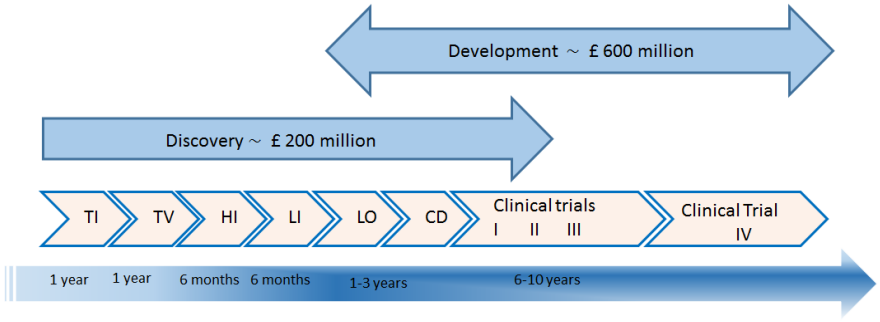


Figure 27. Drug discovery pipeline.

In section 1.7, a body of evidence supports the importance of Bcl-3 as a new molecular target in the treatment of metastatic breast cancer. Now, a brief summary of the hit identification and optimisation phase will show the process to select ten compounds from a commercially available database of thousands molecule using the virtual high-throughput screening.

Initially, the model of the Bcl-3/p50 complex was built by Dr. Andrea Brancale and Dr. Jtka Soukupova, Cardiff University, School of Pharmacy and Pharmaceutical Sciences. Briefly, due to the structural similarity between IkBα and Bcl-3, a Bcl-3/p50 model was build by superposing the ankyrin repeats of Bcl-3 (PDB ID: 1K1A) onto the already published structure of the IkBα-p65/p50 complex (PDB ID:1NFI).

The second molecule of Bcl-3 was added using the pseudo-dyad symmetry of p50 homodimer. The novel model was further refined and stabilised by dynamic simulation with GROMACS (5ns), as published previously (Figure 28).[112]

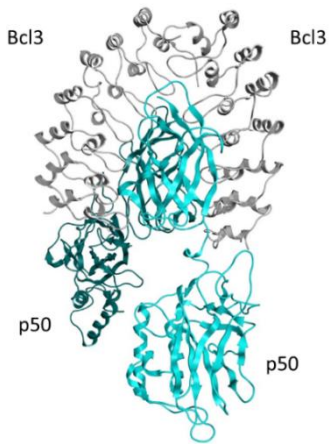


Figure 28. $(Bcl\text{-}3)_2/(p50)_2$ model: in grey, Bcl-3 structure (PDB ID: 1K1A), in cyan and dark cyan p50 subunits.

A novel binding pocket, named MYSGS, at the Bcl-3/p50 interface was discovered and characterised, as depicted in Figure 29.

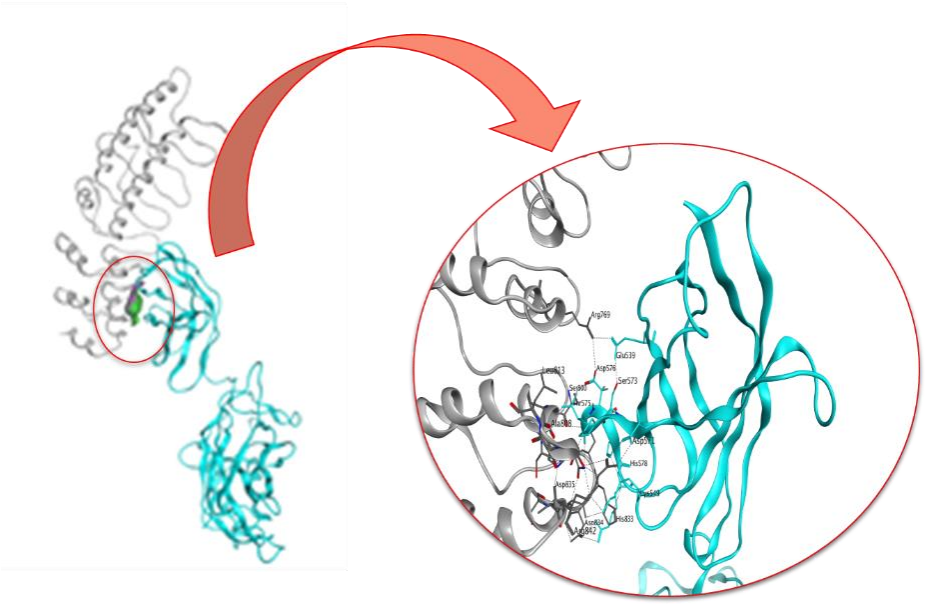


Figure 29. Bcl-3/p50 model: in grey, Bcl-3 structure (PDB ID: 1K1A), in cyan and dark cyan p50 subunits.

The binding pocket has an upper small cavity, mainly constituted at the top of hydrophilic amino acids (such as glutamine, histidine, asparagine) and at the bottom of hydrophobic residues such as, methionine, isoleucine, valine). Below, there is a bigger cavity characterised mainly by hydrophobic residues. The Bcl-3/p50 interactions are stabilised by several hydrogen bonds and hydrophobic interactions, involving several amino acids as reported in Figure 30.

Bcl-3	Arg769 Asp833 Ser800 Lys832 Asp834 His833 Arg842 Asp835 Asn 831
p-50	Glu539 Asp576 Thr575 Asp576 His578 Arg579 Asp571 Lys579 Pro574

Figure 30. Main amino acid residues involved in the Bcl-3/p50 interactions.

As reported in Figure 31, the pharmacophore model was used to search a database of 360 000 commercially available molecules in Specs database: a scoring-based selection reduced the numbers of putative Bcl-3/p50 inhibitors to 121 molecules.

These compounds were evaluated considering two different features: the structural requirements of the binding pocket and the strength of protein-ligand interaction.

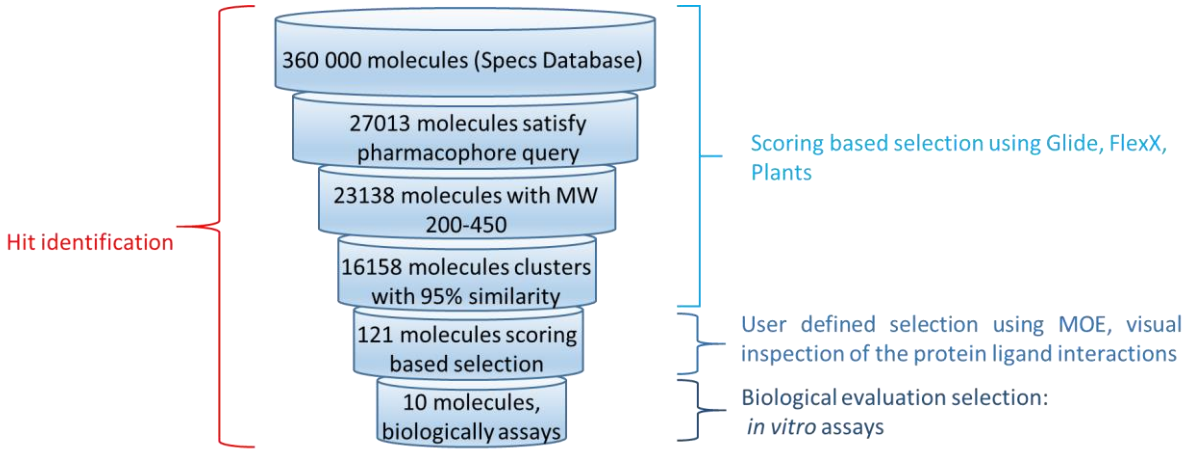
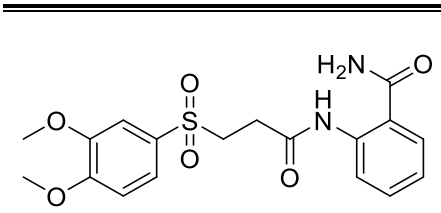
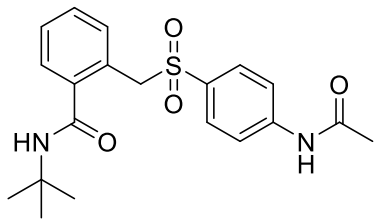
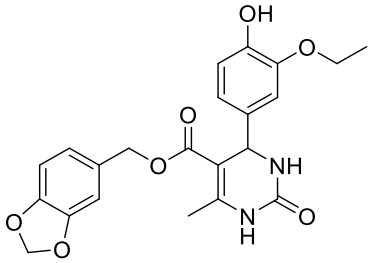


Figure 31.Virtual high throughput screening (vHTS) process.

The virtual screening process ended up with 10 compounds (structure reported in Figure 32), purchased from Specs Database and biologically evaluated in three *in vitro* cell based assays: enzyme-linked immunosorbent assay (ELISA), NF-κB luciferase assay, cell motility.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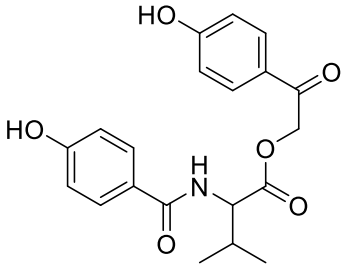	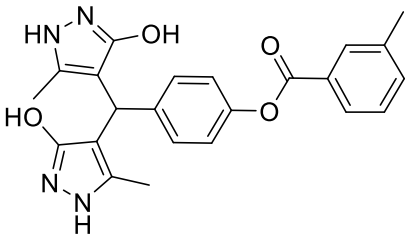	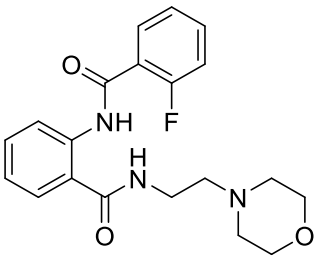
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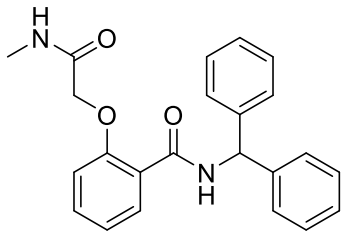
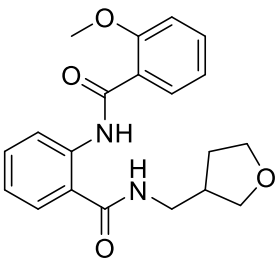
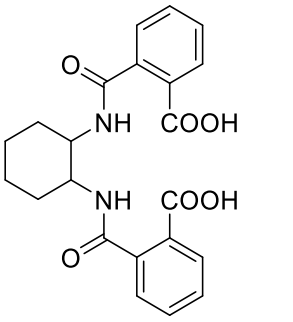
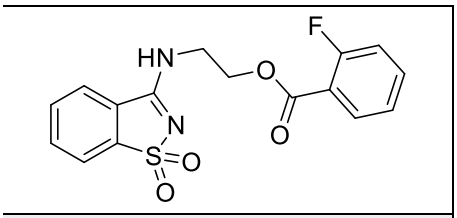
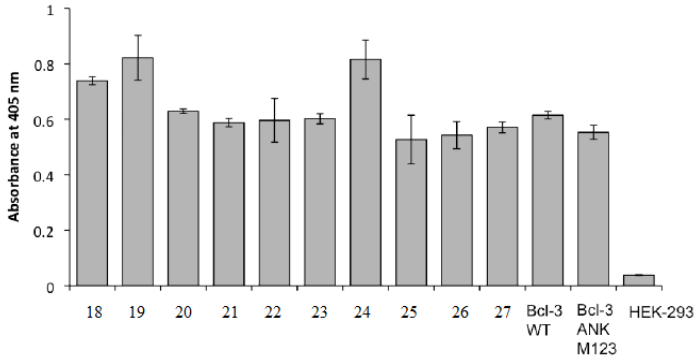
		
24	**25**	**26**



27

Figure 32. Structures of selected ten compounds from high throughput virtual screening.

The outcomes of the ten compounds in the ELISA assay are reported in Figure 33.[112]
Additionally, a small series of analogues of compound **23** was synthesised and compound **15f** (in this thesis, it is reported as compound **186**) resulted to be a promising one. Due to the promising biological results for compound **23** and **186,** in three cell-based assays, they are reported in Table 1.[112]



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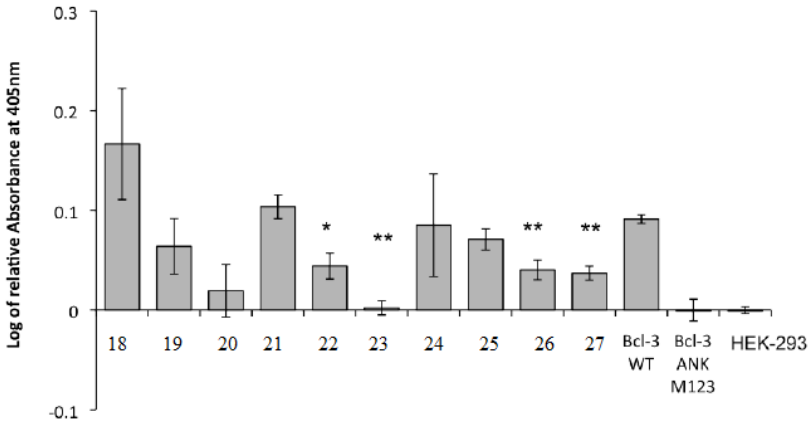

b)

Figure 33. ELISA assay results for the ten compounds selected in the vHTS process. a) Indirect ELISA assay was performed on FLAG coated ELISA plates using Bcl-3 antibody. Absorbance was measured at 405 nm. Error bars represent ±SEM of three independent wells. b) Indirect sandwich ELISA assay was performed on FLAG coated ELISA plates using p50 antibody. Absorbance was measured at 405 nm, normalised to that of Bcl-3 ANK M123 and is plotted on a log scale. Error bars represent ±SEM of six independent wells. (T-test, * = $p<0,05$ and **=$p<0,01$ as compared to Bcl-3-WT).

Table 1. Biological evaluation of the activity of compounds **23** and **186**. * MDA-231 cell line: highly metastatic human basal epithelial cell line.

Compound	ELISA assay (IC50μM)*	NF-kB assay (IC50μM)*	CELL MOTILITY assay (IC50μM)*
23	0.3855	0.04543	0.3104
186	0.06017	0.00697	0.02893

These biological results and the ones in Figure 33 represent the starting point of the actual project with the final aim to individuate a novel clinical candidate, as discussed in section 2.

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Section 2:

OBJECTIVES

2. Objectives

Objective of the project will be the synthesis and biological evaluation of different scaffolds of compounds to identify a novel, putative Bcl-3/p50 inhibitor:

1) synthesis and biological evaluation of analogues of compound **23** (structure reported in Figure 34 and Figure 39). The main modifications carried out are of several types: a) insertion of electron withdrawing and electron donating groups on both A and B rings; b) X may be either a carbonyl group or a sulfonyl group; c) different length of the carbon linker chain (*n* = 2, 3); d) different hetero- and carbo-cycle. These systematic modifications will provide a number of analogues characterised by different lipophilicity, solubility and a variety of different electronic effects eventually stabilizing or destabilizing the analogues in the binding pocket, to assess the contribution of each factor to the biological activity. Synthesised analogues are reported in Table 2.

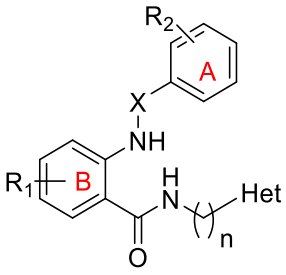


Figure 34. General structure of analogues of compound **23**.

2) Synthesis and biological evaluation of analogues of compound **20** (structure reported in Figure 35 and Figure 40). The main substitutions include: a) modification of R_1, such as the presence of benzyl group or 3, 4 – dimethoxybenzyl group, or the benzo[d][1,3]dioxole group; b) modification of the R_2 with both electron donating and electron withdrawing groups to explore the structure activity relationships of these analogues. Synthesised analogues are reported in Table 3.

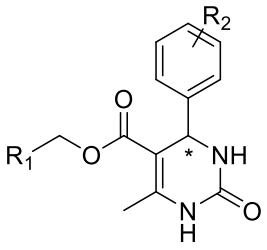
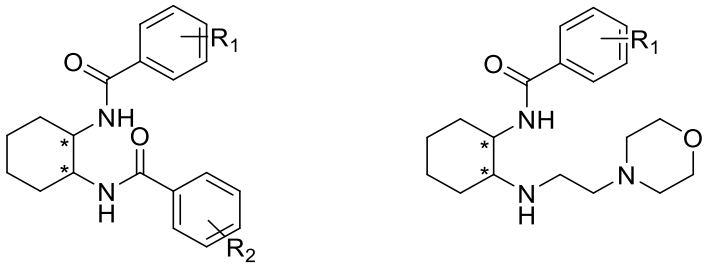


Figure 35. General structure of analogues of compound **20**.

3) Synthesis and biological evaluation of analogues of compound **26** (structure reported in Figure 36 and Figure 41). The main modifications involve the introduction of both electron donating and withdrawing groups in the symmetric (Figure 36.a) and non-symmetric (Figure 36.b) scaffold to investigate a range of logP, logD and electronic effects.
Synthesised analogues are reported in Table 4.



(a) **(b)**

Figure 36. General structure of analogues of compound **26**: a) symmetric scaffold; b) asymmetric scaffold.

4) Synthesis and biological evaluation of analogues of compound **27** (structure reported in Figure 37 and Figure 42). A number of analogues are synthesised introducing different electron donating and electron withdrawing groups in the benzoate ring. Synthesised analogues are reported in Table 5.

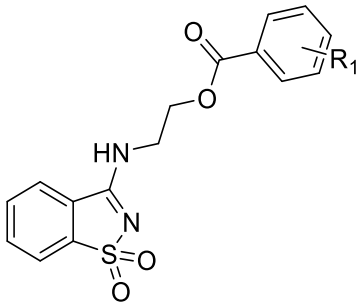
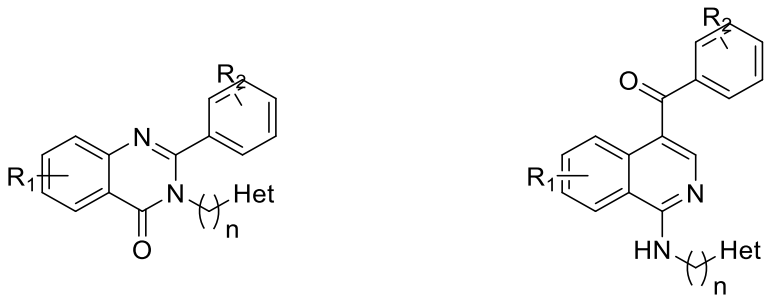


Figure 37. General structure of analogues of compounds **27**.

5) Synthesis and biological evaluation of analogues of compound **23**: closure of the ring according to two different strategies, as reported in Figure 38 and Figure 43. Synthesised analogues are reported in Table 6.



a b

Figure 38. General structure of analogues of compound **23**: a) quinazolinone based scaffold; b) isoquinoline based scaffold.

The biological results will be the driving force to determine structure-activity relationships (SARs) of synthesised compounds to improve the biological activity developing further modifications on the scaffolds and to find a putative, novel clinical candidate.

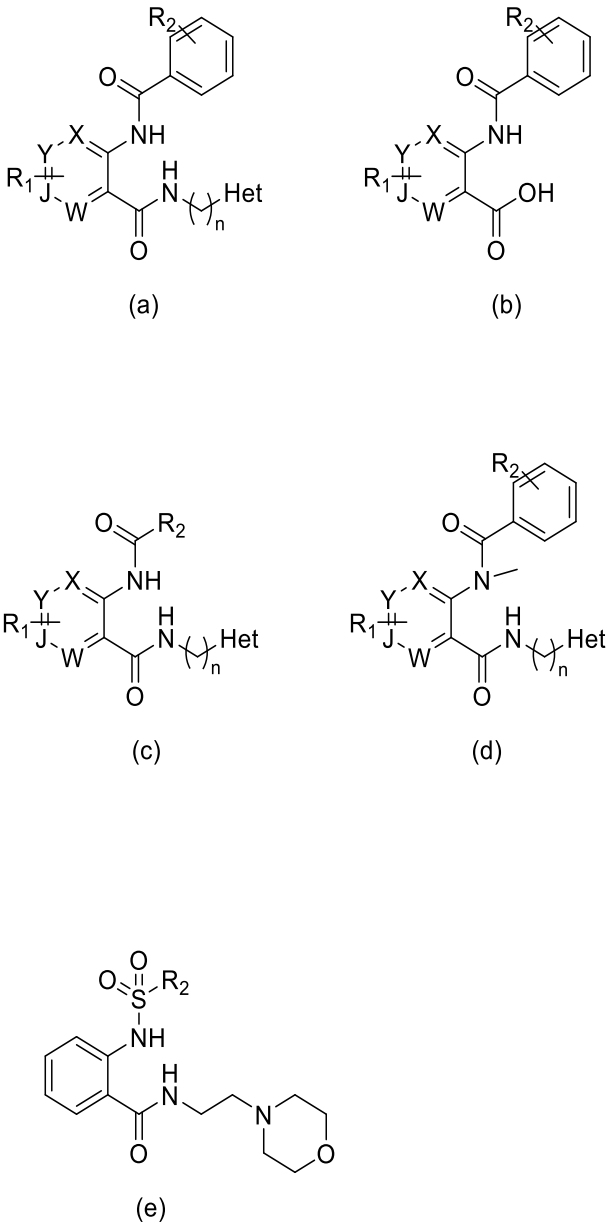


Figure 39. General structure of analogues of compounds **23**.

Table 2. Chemical structure of synthesised compounds **28**-**190**.

Compound	R_1	R_2	Heterocycle	n	X	Y	J	W
28[(a)]	H	2-Cl	Morpholine	2	CH	CH	CH	CH
29[(a)]	H	3-Cl	Morpholine	2	CH	CH	CH	CH
30[(a)]	H	4-Cl	Morpholine	2	CH	CH	CH	CH
31[(a)]	H	2-F,4-OCH_3	Morpholine	2	CH	CH	CH	CH
32[(a)]	H	3-NH_2	Morpholine	2	CH	CH	CH	CH
33[(a)]	H	4-NH_2	Morpholine	2	CH	CH	CH	CH
34[(a)]	4-OCH_3	2-F	Morpholine	2	CH	CH	CH	CH
35[(a)]	2-F	2-F	Morpholine	2	CH	CH	CH	CH
36[(a)]	4-OCH_3	4-OCH_3	Morpholine	2	CH	CH	CH	CH
37[(a)]	2-F	4-OCH_3	Morpholine	2	CH	CH	CH	CH
38[(a)]	2-F	2-F,4-OCH_3	Morpholine	2	CH	CH	CH	CH
39[(a)]	3-OCH_3	4-OCH_3	Morpholine	2	CH	CH	CH	CH
40[(a)]	4-F	4-OCH_3	Morpholine	2	CH	CH	CH	CH
41[(a)]	4-F	2-F	Morpholine	2	CH	CH	CH	CH
42[(a)]	H	2-NO_2	Morpholine	2	CH	CH	CH	CH
43[(a)]	H	3-NO_2	Morpholine	2	CH	CH	CH	CH
44[(a)]	H	4-NO_2	Morpholine	2	CH	CH	CH	CH
45[(a)]	H	2-CF_3	Morpholine	2	CH	CH	CH	CH
46[(a)]	H	3-CF_3	Morpholine	2	CH	CH	CH	CH
47[(a)]	H	4-CF_3	Morpholine	2	CH	CH	CH	CH
48[(a)]	H	4-OCH_3	Morpholine	2	CH	N	CH	CH
49[(a)]	3-OCH_3	2-F	Morpholine	2	CH	CH	CH	CH
50[(a)]	3-OCH_3	3-F	Morpholine	2	CH	CH	CH	CH
51[(a)]	3-OCH_3	4-F	Morpholine	2	CH	CH	CH	CH
52[(a)]	3-OCH_3	2-OCH_3	Morpholine	2	CH	CH	CH	CH
53[(a)]	3-OCH_3	3-OCH_3	Morpholine	2	CH	CH	CH	CH
54[(a)]	3-OCH_3	2,4- OCH_3	Morpholine	2	CH	CH	CH	CH
55[(a)]	H	2-Cl	Phenyl	2	CH	CH	CH	CH
56[(a)]	H	3-Cl	Phenyl	2	CH	CH	CH	CH
57[(a)]	H	4-Cl	Phenyl	2	CH	CH	CH	CH
58[(a)]	H	2-F,4-OCH_3	Phenyl	2	CH	CH	CH	CH
59[(a)]	H	4-OCH_3	Phenyl	2	CH	CH	CH	CH
60[(a)]	H	2-NO_2	Phenyl	2	CH	CH	CH	CH
61[(a)]	H	3-NO_2	Phenyl	2	CH	CH	CH	CH
62[(a)]	H	4- NO_2	Phenyl	2	CH	CH	CH	CH
63[(a)]	H	2-F	Phenyl	2	CH	CH	CH	CH
64[(a)]	4-OCH_3	2-F	Phenyl	2	CH	CH	CH	CH
65[(a)]	2-F	2-F	Phenyl	2	CH	CH	CH	CH
66[(a)]	4-OCH_3	4-OCH_3	Phenyl	2	CH	CH	CH	CH
67[(a)]	2-F	4-OCH_3	Phenyl	2	CH	CH	CH	CH
68[(a)]	3-OCH_3	4-OCH_3	Phenyl	2	CH	CH	CH	CH
69[(a)]	4-F	4-OCH_3	Phenyl	2	CH	CH	CH	CH
70[(a)]	4-F	2-F	Phenyl	2	CH	CH	CH	CH
71[(a)]	3-OCH_3	2-F	Phenyl	2	CH	CH	CH	CH
72[(a)]	3-OCH_3	3-F	Phenyl	2	CH	CH	CH	CH

Compound	R_1	R_2	Heterocycle	n	X	Y	J	W
73[a]	3-OCH_3	4-F	Phenyl	2	CH	CH	CH	CH
74[a]	3-OCH_3	2-OCH_3	Phenyl	2	CH	CH	CH	CH
75[a]	3-OCH_3	3-OCH_3	Phenyl	2	CH	CH	CH	CH
76[a]	3-OCH_3	2,4- OCH_3	Phenyl	2	CH	CH	CH	CH
77[a]	H	4-CF_3	Phenyl	2	CH	CH	CH	CH
78[a]	H	4-OCH_3	Phenyl	2	CH	N	CH	CH
79[a]	H	3-CF_3	Phenyl	2	CH	CH	CH	CH
80[a]	H	3-CF_3	Piperazine	2	CH	CH	CH	CH
81[a]	H	2-CF_3	Piperazine	2	CH	CH	CH	CH
82[b]	H	2-F	-	2	CH	CH	CH	CH
83[b]	H	2-F	-	2	N	CH	CH	CH
84[c]	H	1-Naphtalyl	Morpholine	2	CH	CH	CH	CH
85[c]	H	2-Naphtalyl	Morpholine	2	CH	CH	CH	CH
86[a]	H	2F, 4OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
87[a]	H	4-F	*N*-methyl piperazine	2	CH	CH	CH	CH
88[a]	H	4-OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
89[a]	H	4-CF_3	*N*-methyl piperazine	2	CH	CH	CH	CH
90[c]	H	1-Naphtalyl	Morpholine	3	CH	CH	CH	CH
91[c]	H	2-Naphtalyl	Morpholine	3	CH	CH	CH	CH
92[c]	H	1-Naphtalyl	Phenyl	3	CH	CH	CH	CH
93[a]	H	2-F	*N*-methyl piperazine	3	CH	CH	CH	CH
94[a]	H	1-Naphtalyl	*N*-methyl piperazine	3	CH	CH	CH	CH
95[a]	H	2-Naphtalyl	*N*-methyl piperazine	3	CH	CH	CH	CH
96[c]	H	4-OCH_3	Morpholine	3	CH	CH	CH	CH
97[c]	H	4-OCH_3	Phenyl	3	CH	CH	CH	CH
98[a]	H	4-OCH_3	*N*-methyl piperazine	3	CH	CH	CH	CH
99[a]	H	2-F, 4-OCH_3	Piperazine	2	CH	CH	CH	CH
100[a]	H	3-CF_3	*N*-methyl piperazine	2	CH	CH	CH	CH
101[a]	2-F	4-OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
102[a]	2-F	3-OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
103[a]	2-F	4-OCH_3	Piperazine	2	CH	CH	CH	CH
104[a]	2-F	3-OCH_3	Piperazine	2	CH	CH	CH	CH
105[a]	H	2-F, 4-OCF_3	*N*-methyl piperazine	2	CH	CH	CH	CH
106[a]	H	4-OCH_3	Morpholine	2	CH	CH	N	CH
107[a]	H	4-OCH_3	*N*-methyl piperazine	2	CH	CH	N	CH

Compound	R_1	R_2	Heterocycle	n	X	Y	J	W
108[a]	H	2-CF_3	*N*-methyl piperazine	2	CH	CH	CH	CH
109[a]	H	4-CF_3	Piperazine	2	CH	CH	CH	CH
110[d]	H	2-F	Morpholine	2	CH	CH	CH	CH
111[c]	3-OCH_3	2-naphthayl	Phenyl	3	CH	CH	CH	CH
112[c]	3-OCH_3	2-naphthayl	Morpholine	3	CH	CH	CH	CH
113[c]	3-OCH_3	2-naphthayl	*N*-methyl piperazine	3	CH	CH	CH	CH
114[a]	4, 5-OCH_3	4-OCH_3	Morpholine	3	CH	CH	CH	CH
115[a]	3, 4, 5-OCH_3	4-OCH_3	Morpholine	3	CH	CH	CH	CH
116[c]	3, 4, 5-OCH_3	3-pyridyl	Morpholine	3	CH	CH	CH	CH
117[c]	H	3-pyridyl	Phenyl	3	CH	CH	CH	CH
118[c]	H	3-pyridyl	Morpholine	3	CH	CH	CH	CH
119[a]	H	2-F	Morpholine	3	CH	CH	CH	CH
120[a]	H	2-F	Phenyl	3	CH	CH	CH	CH
121[c]	H	2-naphthayl	Phenyl	3	CH	CH	CH	CH
122[a]	H	2-(methylthio)	Morpholine	2	CH	CH	CH	CH
123[a]	H	2-F, 4-benzyloxy	Morpholine	2	CH	CH	CH	CH
124[a]	5-NO_2	2-F	Morpholine	2	CH	CH	CH	CH
125[a]	5-NH_2	2-F	Morpholine	2	CH	CH	CH	CH
126[a]	H	4-OH	Phenyl	3	CH	CH	CH	CH
127[a]	H	2-F, 4-OH	Morpholine	2	CH	CH	CH	CH
128[a]	3-OCH_3	2-naphthayl	Phenyl	2	CH	CH	CH	CH
129[a]	4-OCH_3	2-naphthayl	Phenyl	3	CH	CH	CH	CH
130[a]	3, 4, 5-OCH_3	2-F	Phenyl	3	CH	CH	CH	CH
131[a]	3-OCH_3	1-naphthayl	Phenyl	3	CH	CH	CH	CH
132[a]	4-OCH_3	2-naphthayl	Morpholine	3	CH	CH	CH	CH
133[a]	3-OCH_3	1-naphthayl	Morpholine	3	CH	CH	CH	CH
134[c]	2-F	2-nicotinyl	Morpholine	3	CH	CH	CH	CH
135[a]	4-OCH_3	2-F	Phenyl	3	CH	CH	CH	CH
136[c]	2-F	2-nicotinyl	*N*-methyl piperazine	3	CH	CH	CH	CH
137[c]	2-F	2-nicotinyl	Phenyl	3	CH	CH	CH	CH
138[a]	3, 4, 5-OCH_3	4-OCH_3	*N*-methyl piperazine	3	CH	CH	CH	CH
139[c]	4, 5-OCH_3	2-nicotinyl	Morpholine	3	CH	CH	CH	CH
140[a]	4, 5-OCH_3	2-F	Morpholine	3	CH	CH	CH	CH
141[a]	4, 5-OCH_3	2-F	Phenyl	3	CH	CH	CH	CH
142[a]	H	4-methoxy methoxy	Phenyl	3	CH	CH	CH	CH

Compound	R_1	R_2	Heterocycle	n	X	Y	J	W
143[c]	3-OCH_3	3-nicotinyl	Morpholine	3	CH	CH	CH	CH
144[c]	H	2-(methylthio)-	Morpholine	3	CH	CH	CH	CH
145[c]	3-OCH_3	2-nicotinyl	*N*-methyl piperazine	3	CH	CH	CH	CH
146[c]	3-OCH_3	1-naphthayl	*N*-methyl piperazine	3	CH	CH	CH	CH
147[a]	H	2, 3-F, 4-OCH_3	Morpholine	3	CH	CH	CH	CH
148[a]	4, 5-OCH_3	2-F	*N*-methyl piperazine	3	CH	CH	CH	CH
149[a]	H	4-NO_2	Morpholine	3	CH	CH	CH	CH
150[a]	H	4-NO_2	*N*-methyl piperazine	3	CH	CH	CH	CH
151[a]	H	4-NO_2	Phenyl	3	CH	CH	CH	CH
152[a]	H	2, 3-F, 4-OCH_3	*N*-methyl piperazine	3	CH	CH	CH	CH
153[a]	H	2, 3-F, 4-OCH_3	Phenyl	3	CH	CH	CH	CH
154[c]	3-OCH_3	2-nicotinyl	Phenyl	3	CH	CH	CH	CH
155[c]	4-F	2-nicotinyl	Phenyl	3	CH	CH	CH	CH
156[c]	4-F	2-nicotinyl	Morpholine	3	CH	CH	CH	CH
157[c]	H	2-nicotinyl	Morpholine	2	CH	CH	CH	CH
158[c]	H	2-nicotinyl	*N*-methyl piperazine	2	CH	CH	CH	CH
159[a]	H	2-OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
160[a]	H	2-F	Piperazine	2	CH	CH	CH	CH
161[a]	H	4-OCH_3	Piperazine	2	CH	CH	CH	CH
162[a]	H	4-NO_2	*N*-methyl piperazine	2	CH	CH	CH	CH
163[a]	H	H	*N*-methyl piperazine	2	CH	CH	CH	CH
164[a]	H	2-F, 6-F	*N*-methyl piperazine	2	CH	CH	CH	CH
165[a]	H	3-F, 5-F	*N*-methyl piperazine	2	CH	CH	CH	CH
166[a]	H	3-F, 5-F	Piperazine	2	CH	CH	CH	CH
167[a]	H	2-F, 4-F	*N*-methyl piperazine	2	CH	CH	CH	CH
168[a]	H	4-NO_2	Piperazine	2	CH	CH	CH	CH
169[a]	H	2-F, 4-F	Piperazine	2	CH	CH	CH	CH
170[a]	H	3-Cl	*N*-methyl piperazine	2	CH	CH	CH	CH
171[a]	2, 3, 4-OCH_3	2-F	Morpholine	2	CH	CH	CH	CH

Compound	R_1	R_2	Heterocycle	n	X	Y	J	W
172[a]	4, 5-OCH_3	2-F	Morpholine	2	CH	CH	CH	CH
173[a]	2, 3, 4-OCH_3	4-OCH_3	Morpholine	2	CH	CH	CH	CH
174[c]	4, 5-OCH_3	2-nicotinyl	*N*-methyl piperazine	2	CH	CH	CH	CH
175[a]	4, 5-OCH_3	2-F	*N*-methyl piperazine	2	CH	CH	CH	CH
176[a]	2, 3, 4-OCH_3	2-F	*N*-methyl piperazine	2	CH	CH	CH	CH
177[a]	4, 5-OCH_3	4- OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
178[a]	2, 3, 4-OCH_3	4-OCH_3	*N*-methyl piperazine	2	CH	CH	CH	CH
179[c]	2, 3, 4-OCH_3	2-nicotinyl	Morpholine	2	CH	CH	CH	CH
180[c]	H	2-nicotinyl	Phenyl	2	CH	CH	CH	CH
181[a]	H	4-Cl	*N*-methyl piperazine	2	CH	CH	CH	CH
182[a]	H	3-Cl	Piperazine	2	CH	CH	CH	CH
183[a]	H	2-F	*N*-methyl piperazine	2	CH	CH	CH	CH
184[a]	H	2, 5-F	Piperazine	2	CH	CH	CH	CH
185[a]	2, 3, 4-OCH_3	2-nicotinyl	*N*-methyl piperazine	2	CH	CH	CH	CH
186[a]	H	4-OCH_3	Morpholine	2	CH	CH	CH	CH
187[e]		4-OCH_3	Morpholine	2	CH	CH	CH	CH
188[e]	-	4-Cl	Morpholine	2	CH	CH	CH	CH
189[e]	-	2-F	Morpholine	2	CH	CH	CH	CH
190[e]	-	2, 4-F	Morpholine	2	CH	CH	CH	CH

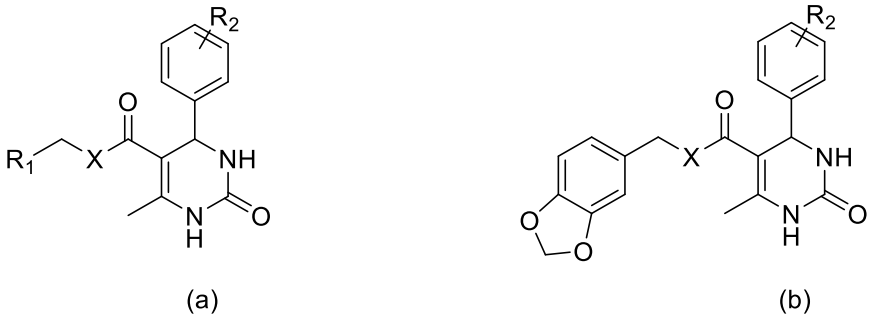


Figure 40. General structure of analogues of compounds **20**.

Table 3. Chemical structure of synthesised compounds **191** - **210**.

Compound	**R_1**	**R_2**
191[(a)]	Benzyl	4-OCH_3
192[(a)]	Benzyl	4-OH
193[(a)]	Benzyl	3,4,5-OCH_3
194[(a)]	Benzyl	2,5-OCH_3
195[(a)]	Benzyl	2-OCH_3
196[(a)]	Benzyl	3-OCH_3
197[(a)]	Benzyl	4-CF_3
198[(a)]	Benzyl	3-OEt
199[(a)]	Benzyl	2-F, 4-OCH_3
200[(a)]	3,4-dimethoxy benzyl	4-OCH_3
201[(a)]	3,4-dimethoxy benzyl	4-OH
202[(a)]	3,4-dimethoxy benzyl	3,4,5-OCH_3
203[(a)]	3,4-dimethoxy benzyl	3-OCH_3
204[(a)]	3,4-dimethoxy benzyl	4-CF_3
205[(a)]	3,4-dimethoxy benzyl	3-OEt
206[(a)]	3,4-dimethoxy benzyl	2-F, 4-OCH_3
207[(a)]	3,4-dimethoxy benzyl	2-OCH_3
208[(a)]	3,4-dimethoxy benzyl	4-Cl
209[(b)]	benzo[d][1,3]dioxol-5-ylmethyl	4-Cl
210[(b)]	benzo[d][1,3]dioxol-5-ylmethyl	2-F, 4-OCH_3

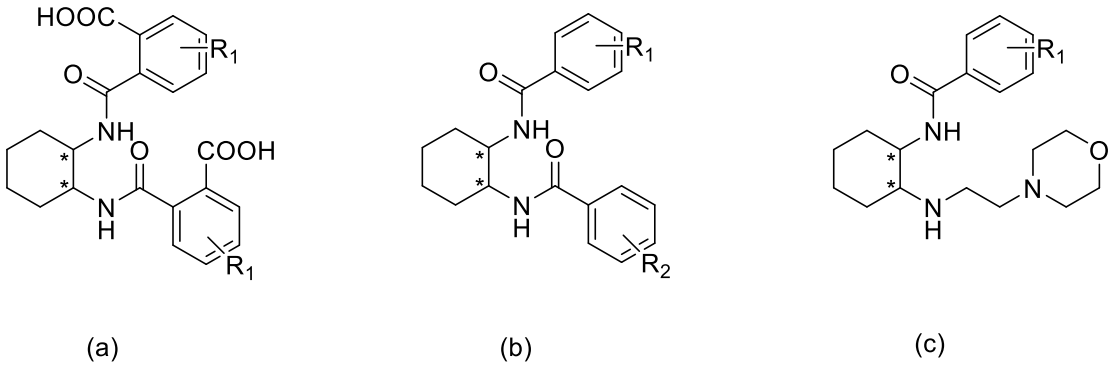


Figure 41. General structure of analogues of compounds **26**.

Table 4. Chemical structure of synthesised compounds **211**-**218**.

Compound	**Chirality**	**R_1**	**R_2**
211[(a)]	mixture[*]	4, 5- Cl	-
212[(b)]	R, R	2-F	2-F
213[(b)]	S, S	2-F	2-F
214[(b)]	R, R	3-F	3-F
215[(b)]	S, S	3-F	3-F
216[(b)]	R, R	2-F, 4-OCH_3	2-F, 4-OCH_3
217[(b)]	S, S	2-F, 4-OCH_3	2-F, 4-OCH_3
218[(c)]	R,R	2-F	-

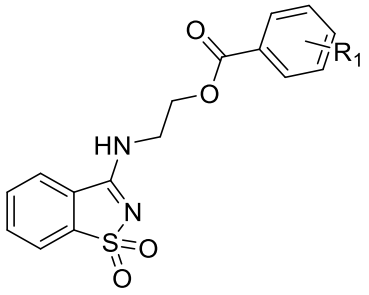


Figure 42. General structure of analogues of compound **27**.

Table 5. Chemical structure of synthesised compounds **219**-**221**.

Compound	**R_1**
219	3-F
220	4-F
221	4-OCH_3

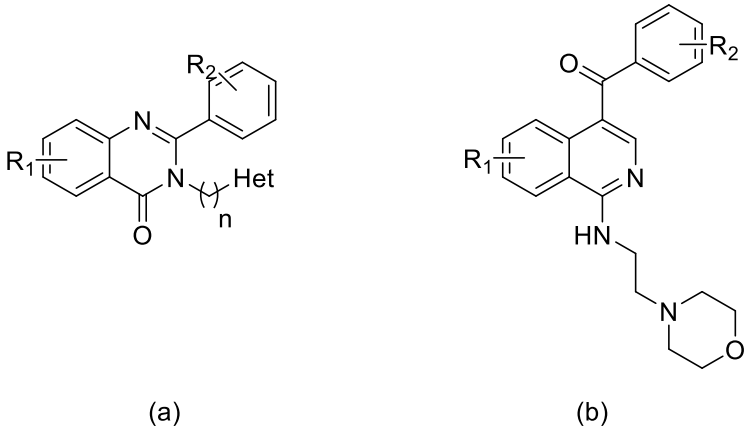
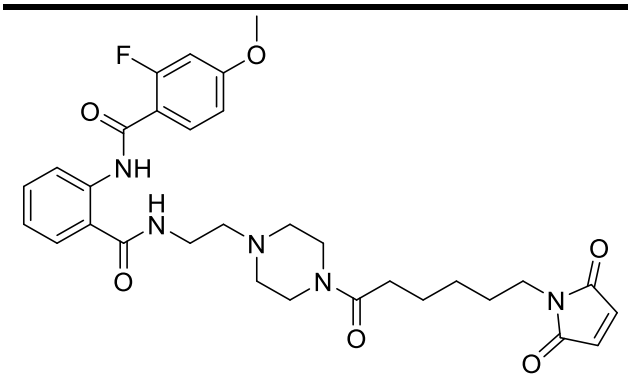


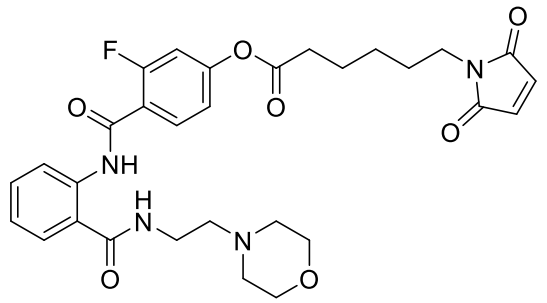
Figure 43. General structure of compounds **23**: closure of central ring.

Table 6. Chemical structure of synthesised compounds **222**-**226**.

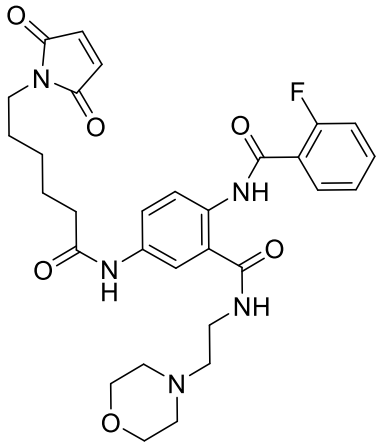
Compound	**R1**	**R_2**	**Heterocycle**	**n**
222[(a)]	-	2-F, 4-OCH_3	Morpholine	2
223[(a)]	-	2-F, 4-OCH_3	*N*-methyl piperazine	2
224[(a)]	-	4-OCH_3	Morpholine	2
225[(a)]	-	2-F	Morpholine	2
226[(b)]	-	2-F	-	-



Compound 227



Compound 228



Compound 229

Figure 44. General structure of modified compounds of analogues **23** to insert a Michael acceptor moiety.

Section 3:

SYNTHESIS

3.1 First scaffold: analogues of compound 23 (compounds 28-190)

By means of retrosynthetic pathway analysis (Figure 45), it is possible to individuate the building blocks of analogues **28-186**.

The synthesised compounds are characterised by the presence of three cycles:

1. phenyl ring (A);
2. benzamide ring (B);
3. heterocycle (Het), bound to the molecule by a *n* carbon linker chain.

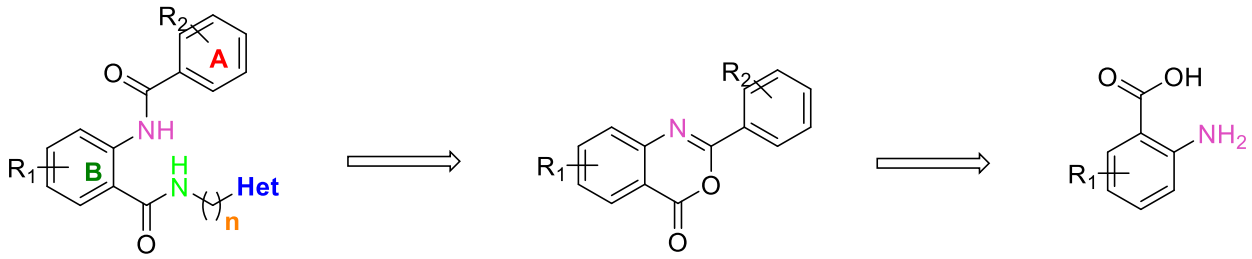
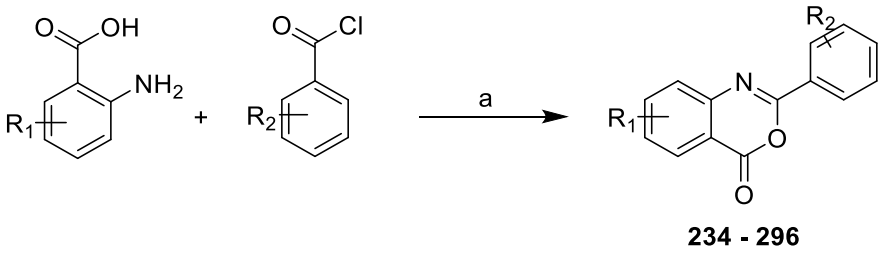


Figure 45. Retrosynthetic pathway for analogues of compound **23**.

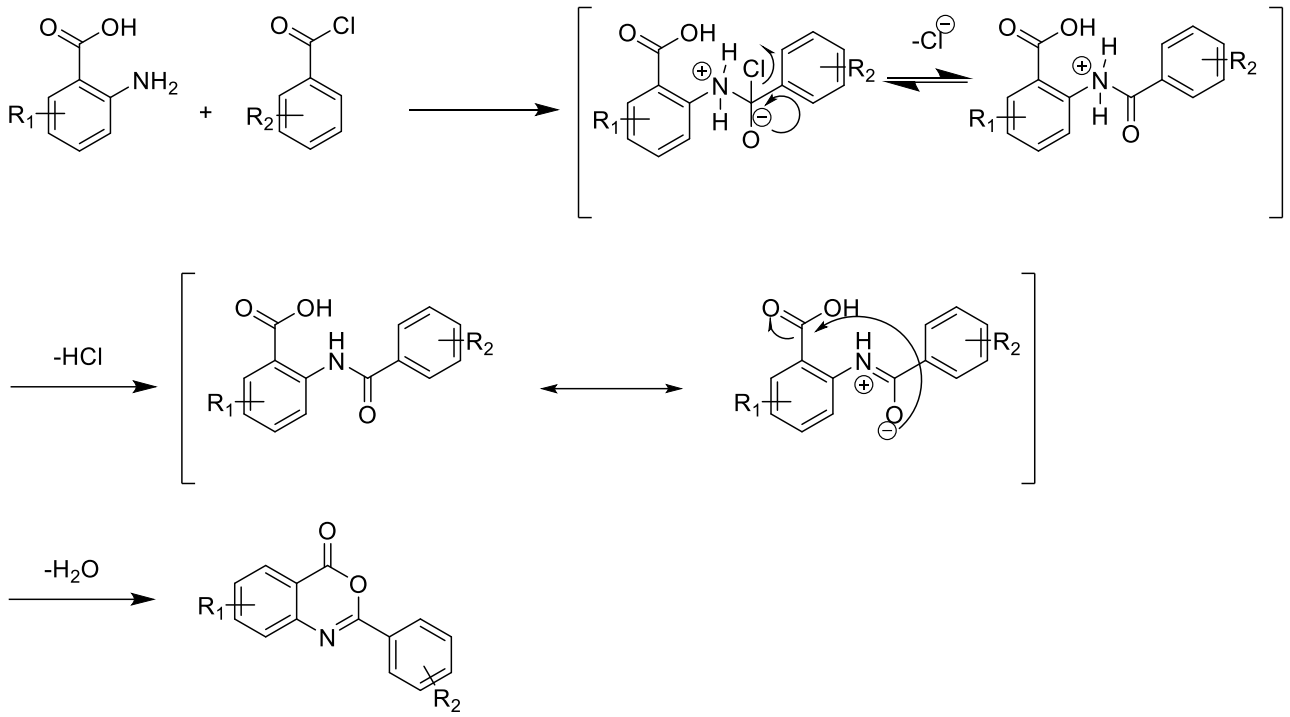
The synthetic strategy provides for a two-step synthetic route under mild conditions: the former step, as represented in *scheme*$^{(1)}$ involves nucleophilic acyl substitution and cyclo-condensation reaction by the amine group of the anthranilic acid on benzoyl chloride, in pyridine at room temperature. The reaction provided the intermediates in high yields.[1]

Various substituents were introduced on both ring A and B, as shown in section 2 (table 2, figure 36).



Scheme$^{(1)}$. Reagents and reaction condition: **a)** pyridine, 25 °C, 2-8 hours, yield: 25-96%.

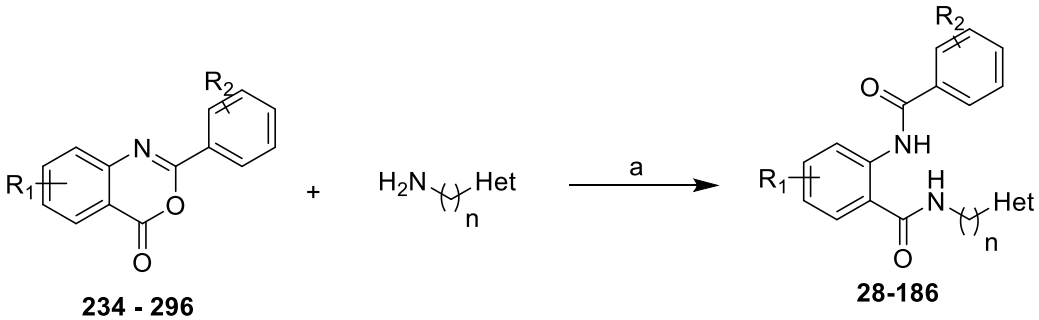
The postulated mechanism of the reaction is shown in *scheme*$^{(2)}$:



Scheme[2]. Mechanism of nucleophilic acyl substitutions and cyclo-condensation reaction between anthranilic acid derivatives and benzoyl chloride.

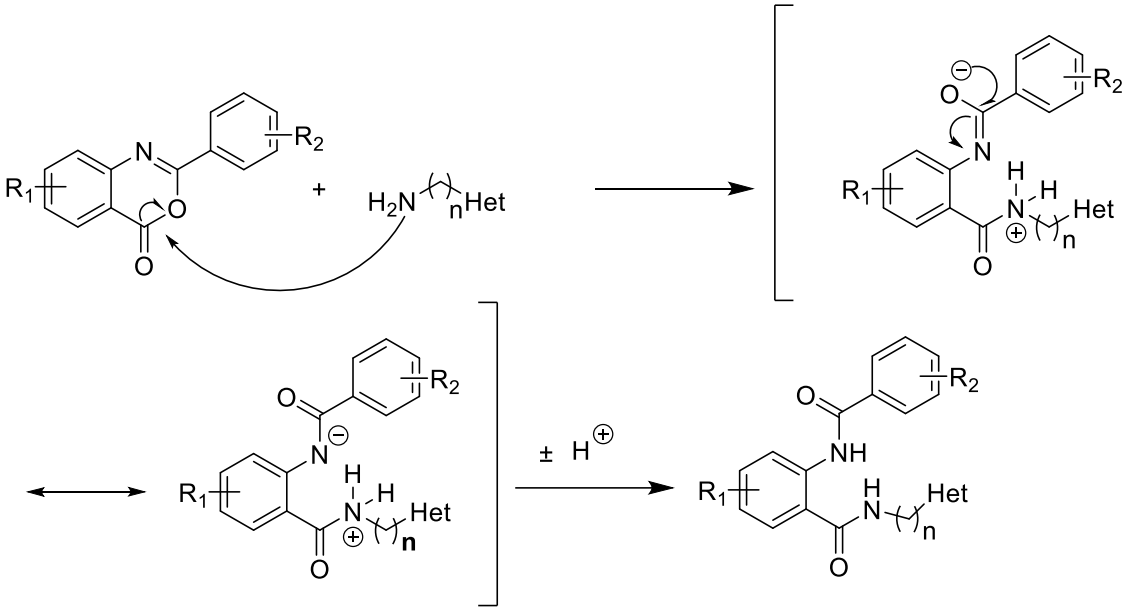
The second step of reaction involves the nucleophilic attack of amine on the carboxylic moiety of the closed intermediate, 2-phenyl-4H-benzo[d][1,3]oxazin-4-one, to obtain the final opened compound, using *N*,*N*-diisopropylamine (DIPEA) as Hunig's base in *N*,*N*-dimethyl-formamide (DMF) as polar aprotic solvent co-adjuvanting the nucleophilic acyl substitution, as displayed in *scheme*[3].

To explore a variety of different features such as lipophilicity, solubility and acidity, morpholine ring in compound **23** was replaced by different hetero-cycle, such as piperazine, *N*-methyl-piperazine and aromatic carbo-cycle, such as phenyl ring. In addition, a different length for the carbon linker chain (n = 1, 2, 3) was also explored.



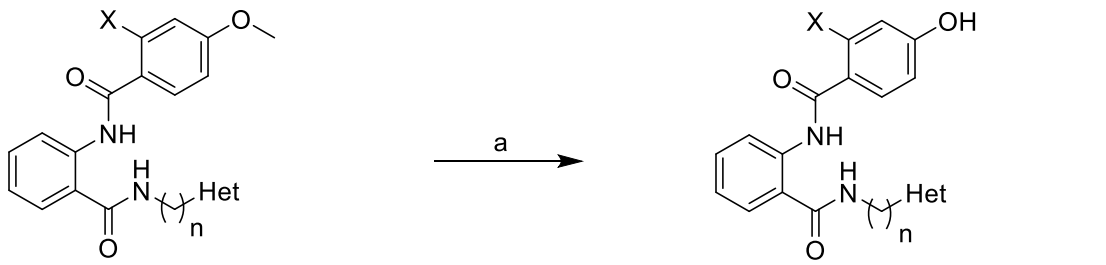
Scheme[3]. *Reagents and reaction condition:* **a)** DMF, DIPEA, 25°C, 4 - 16 hours; yield: 24-85%

The postulated mechanism of reaction is shown in *scheme*[(4)]:



Scheme[(4)]. Mechanism of reaction for the nucleophilic substitution.

Compound **123** was synthesised starting from the 2-fluoro-4-benzyloxy-benzoic acid according to the usual synthetic route, as described above. O-Debenzylation of phenol ether was performed by hydrogenolysis with H_2/Pd and cleavage by chlorosulfonyl isocyanate-sodium hydroxide in order to obtain compound **127**.[2] Only the unreacted starting material was re-collected. Hence, a different strategy was applied. Demethylation of aryl methyl ether in presence of boron tribromide (BBr_3) for compounds **31**, **97** yields to compounds **126**, **127** in good yields.[3]



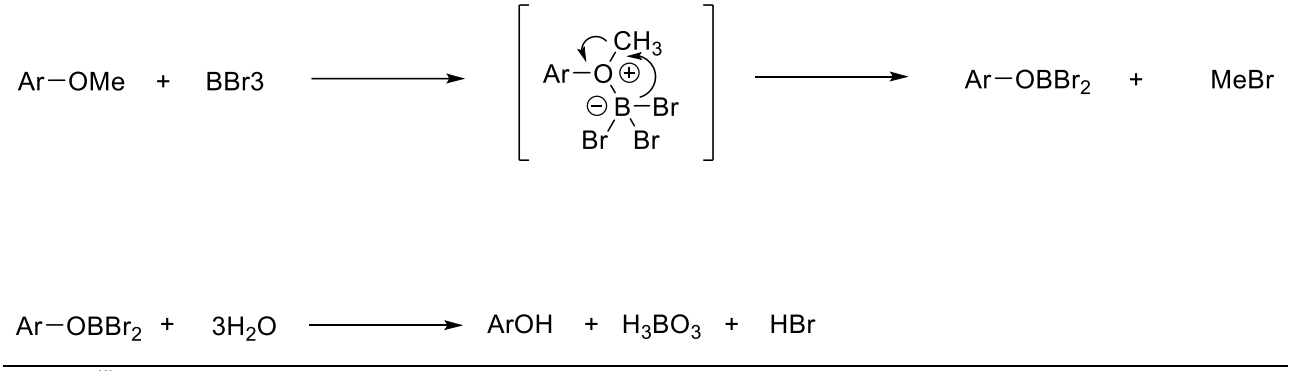
(97: X=H, n=3, Het=phenyl)
(31: X=F, n=2, Het=morpholine)

(126: X=H, n=3, Het=phenyl)
(127: X=F, n=2, Het=morpholine)

Scheme[(5)]. *Reagents and reaction condition:* **a)** BBr_3, anhydrous dichloromethane, -78 °C, 12 hours; yield: 38-84%.

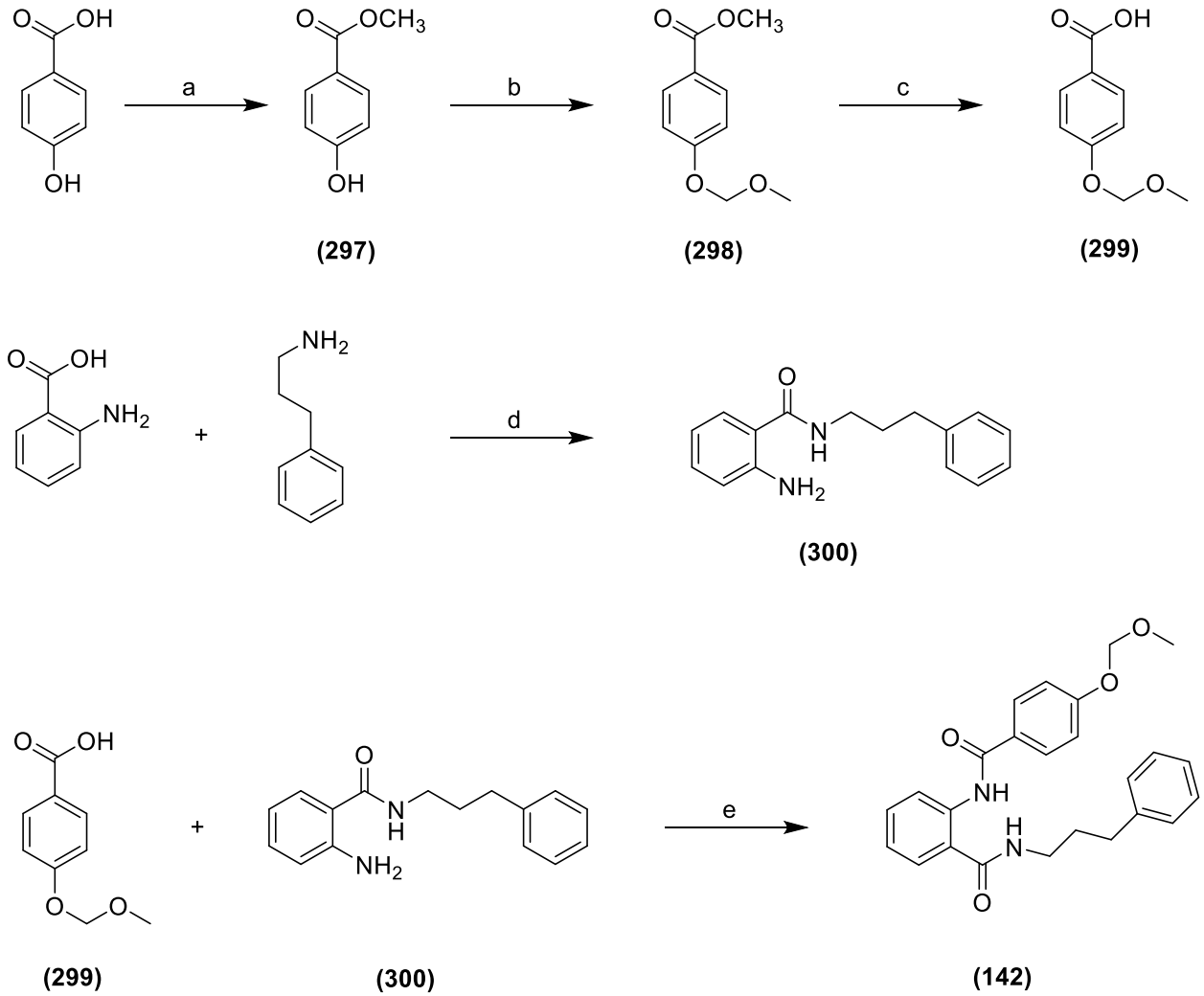
The Lewis acidic boron centre forms an ether adduct with the oxygen and loses one bromine which is now free to nucleophilically attack the methyl group and cleaving the C-O bond as shown in *scheme*[(6)]**.** The di-bromo (phenoxy) borane is hydrolysed upon aqueous work-up to furnish the

desired compound.



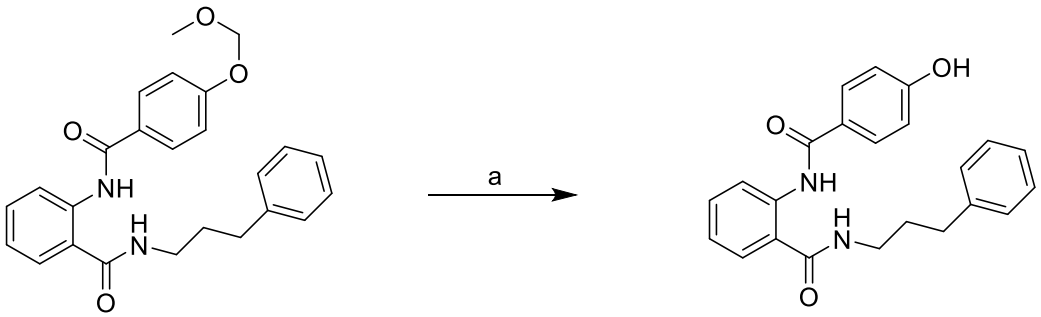
Scheme[6]. Postulated mechanism for the cleavage of methyl aryl ethers.

Compound **142** was synthesised using a different approach, as reported on *scheme*[7].



Scheme[7]. *Reagents and reagent condition:* **a)** MeOH, HCl 12N, 80 °C, 24 hours, yield: 82%; **b)** MOM-Cl, DIPEA, dichloromethane, 0 °C → 25 °C, 5 hours, yield: 84%; **c)** NaOH 1M, 1,4-dioxane, 100 °C, 3 hours, yield: 78%; **d)** EDCI, HOBt, dichloromethane, 25 °C, 15 hours, yield: 56%; **e)** EDCI, HOBt, dichloromethane, 25 °C, 24 hours, yield: 26%.

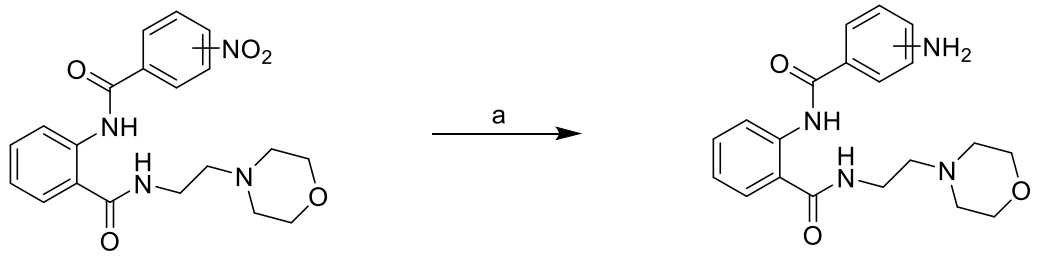
Derivatisation of the carboxylic acid moiety by methyl ester formation (compound **297**) is followed by protection of the phenolic –OH moiety using the chloromethyl methyl ether (MOM) protecting group (compound **298**).[4] The alkaline hydrolysis of esters produces the carboxylic acid (compound **299**) which is free to be *in situ* activated by hydroxybenzotriazole (HOBT) to generate in the fourth step (d) of the synthetic route the 2-benzamidobenzoic acid (compound **300**).[5] The activated ester strategy affords formation of the desired final compound **142** in mild condition and in good yield.
The MOM deprotection[4] was performed on compound **142** as described in *scheme(8)*:



(142) **(126)**

Scheme(8). Reagents and reagent condition: **a)** HCl 37%, MeOH, 25° C, 20h, yield: 45%.

Compound **32** and **33** were synthesised by catalytic hydrogenation in presence of carbon on palladium catalyst, at room temperature for 24 hour,[6] as reported in *scheme(9)*.

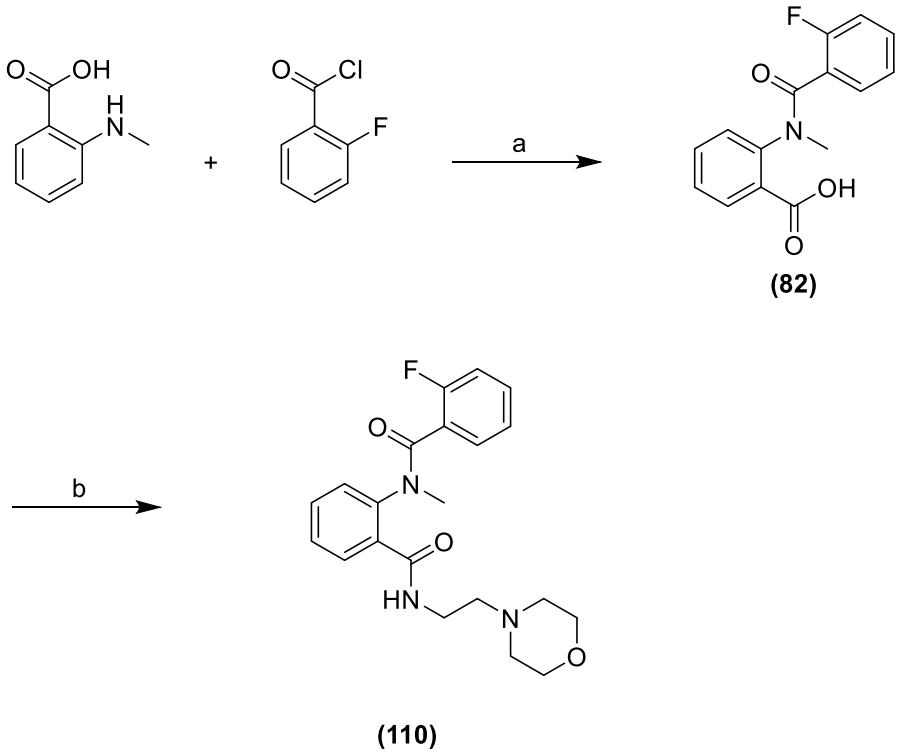


(43, 44) **(32, 33)**

Scheme(9). Reagents and reagent condition: **a)** H_2 balloon, CH_3OH, Pd/C, 25 °C, 16 hours, yield: 38-46%.

Due to the low yields achieved with the H_2/(Pd/C) balloon method, reduction of the nitro group in compound **124** to amino group in compound **125** was performed with tin(II) chloride in ethyl acetate: compound **125** was obtained in really good yield (59%) compared to compounds **32** and **33**.
The synthetic route for the synthesis of compound **110** involves a slightly different strategy, as reported in *scheme(10)*. Compound **110** was synthesised from 2-(methyl-amino)-benzoic acid in

presence of 2-fluoro benzoyl chloride in pyridine to produce the 2-(2-fluoro-*N*-methylbenzamido)-benzoic acid intermediate (compound **82**). Upon acidification with hydrochloric acid, the desired compound (**82**) precipitated. Reactivity of the benzoic acid intermediate (**82**) in the final nucleophilic acyl substitution was then enhanced with the activated ester strategy: 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) was used for the *N*-acylation with 2-morpholinoethan-1-amine in *N*, *N*-dimethylformamide and in the presence of *N*, *N*- diisopropylethylamine to afford isolation of the final methylated compound (**110**).

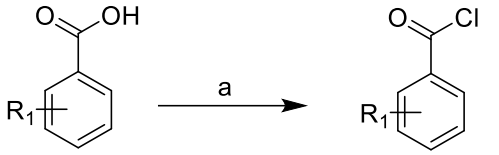


Scheme [10]. Reagents and reagent condition: **a)** pyridine, HCl, 0 → 25 °C, 6 hours, yield: 37%; **b)** 4-(2-Aminoethyl)morpholine, HATU, DIPEA, DMF, 25 °C, 8 hours, yield: 28%.

For final compounds **31** and **123**, the starting benzoyl chlorides were not commercially available: they were synthesised starting from the corresponding benzoic acid.

A widely method for the conversion of benzoic acid into more reactive benzoyl chloride uses thionyl chloride since by-products of this reaction are gases (sulphur dioxide and hydrochloric acid), hence they are eliminated heating the reaction mixture at 85 °C (*scheme [11]*). An excess of thionyl chloride, using *N*, *N*-dimethyl-formamide in catalytic amount allows the formation of the desired compounds with a good yield (approximately 70%). The use of DMF as a catalyst allows the formation of the Vilsmeier reagent: the iminium double bond provides an additional

electrophilic character on the carboxylic acid.

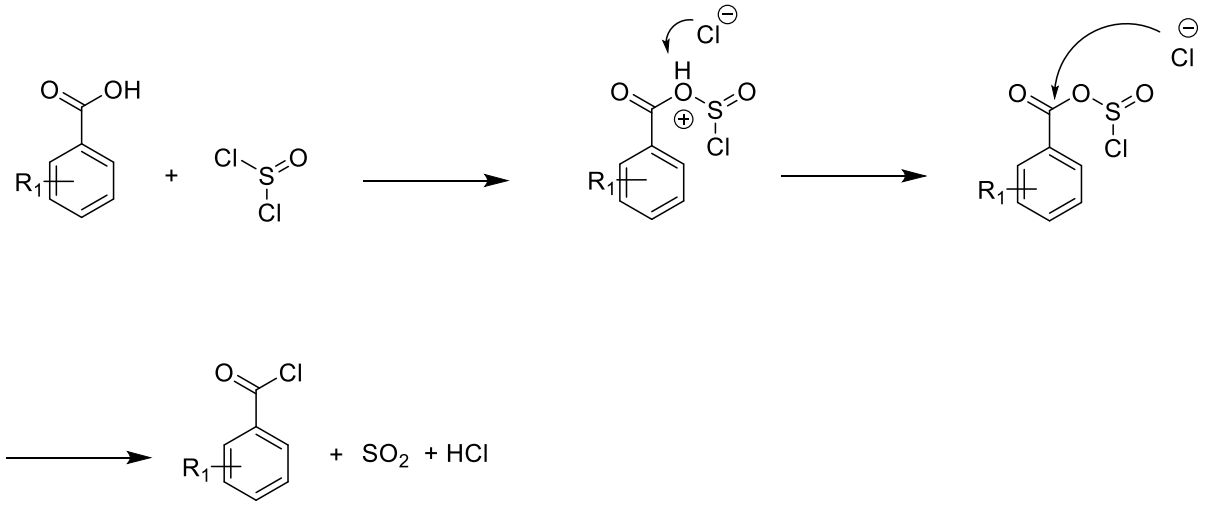


(230, 231)

Scheme(11). Reagents and reagent conditions: **a)** $SOCl_2$, DMF, 85 °C, 8 hours.

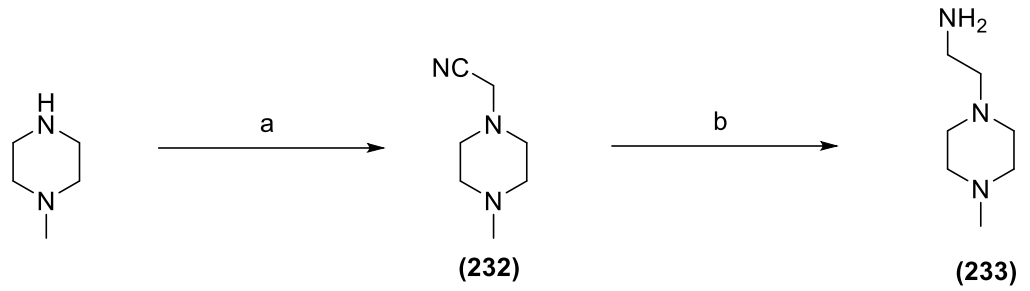
The excess of thionyl chloride was removed by distillation under reduced pressure. The crude compound was used in the next step of reaction without further purification.[2]

The mechanism of the acid chloride formation is shown in *scheme(12)*:



Scheme(12). Mechanism of chlorination using thionyl chloride.

To replace the morpholine ring with the *N*-methyl piperazine ring, it was then necessary to synthesise the 2-(4-methylpiperazi-*N*-1-yl) ethanamine (compound **233**). As shown in *scheme(13)*, alkylation of the *N*-methyl piperazine to introduce the nitrile functional group is followed by reduction with lithium aluminium hydride.[3] The first step of reaction is a S_{N2} reaction of secondary amine with primary alkyl nitrile in presence of a base. The second step is the reduction of the nitrile group using the lithium aluminium hydride as source of nucleophile hydride to obtain the primary amine.



Scheme[13]. Reagents and reagent condition: **a)** K_2CO_3, acetonitrile, chloroacetonitrile, 25 °C; **b)** $LiAlH_4$, diethyl ethere/tetrahydrofuran, 25 °C, 24 h.

The overall yield of the reaction dropped because 2-(4-methylpiperazin-1-yl) acetonitrile is not soluble neither in tetrahydrofuran nor in ethyl ether, which are the most common and unreactive solvents used in this reaction.

The carbonyl group of the benzamide attached to A ring (Figure 32) may be replaced by the isosteric sulfonyl moiety. General structure of analogues **187** – **190** is reported in Figure 46.

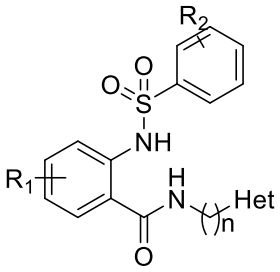
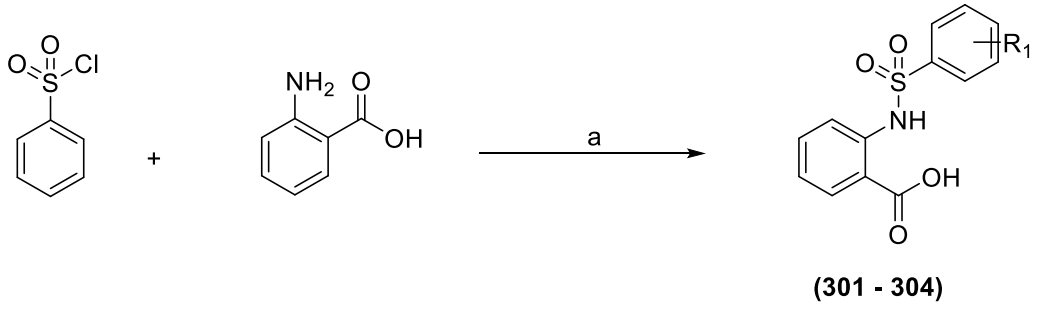


Figure 46. General structure of compounds **187** – **190**.

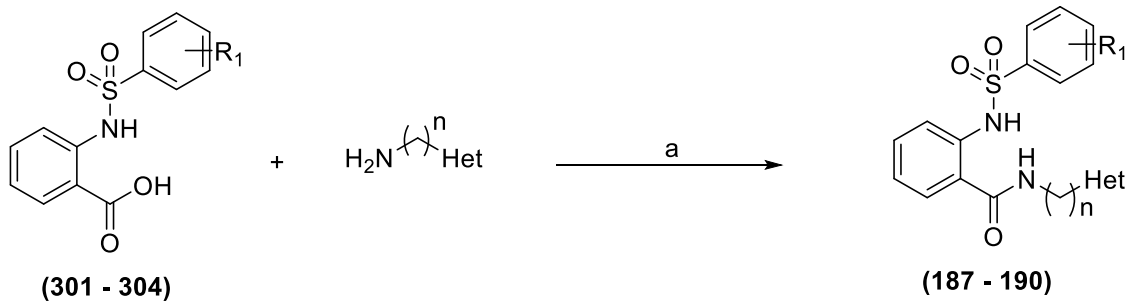
The synthetic strategy for this scaffold is a two-step synthetic route: the first step (*scheme[14]*) of reaction involves the formation of the 2-(phenyl sulfonamide) benzoic acid by nucleophilic acyl substitution reaction of the anthranilic acid with sulfonyl chloride in sodium hydroxide aqueous solution.



Scheme[14]. Reagents and reagent condition: **a)** NaOH 2M, 1-3 hours, 25 °C, yield: 34 – 65%.

Quenching the reaction with hydrochloric acid 1M allows to collect the desired compound as a

precipitate. The second step of reaction, in *scheme*[(15)], is a nucleophilic acyl substitution in which the carboxylic acid mojety is activated by formation of highly reactive, activated esters using the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and hydroxy benzotriazole (HOBt).[13]

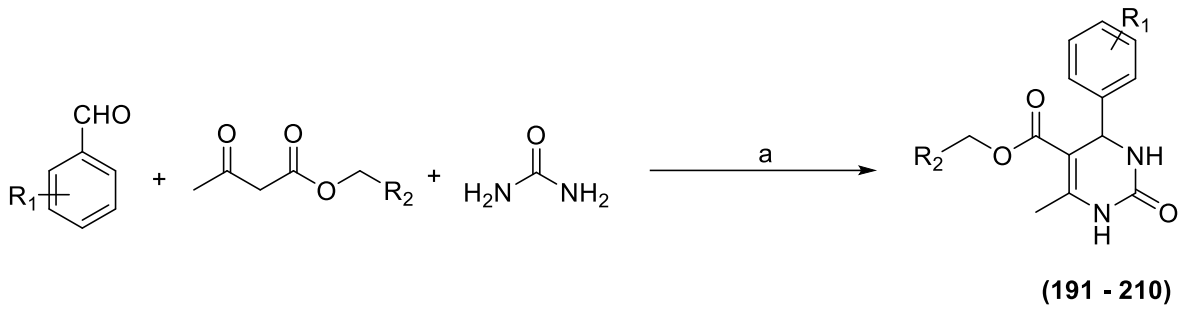


Scheme[(15)]. *Reagents and reagent condition:* **a)** EDCI, HOBt, CH_2Cl_2, DMAP, 12-24 hours, 25 °C, yield: 26-61 %.

Carboxylic acids are poorly reactive and thus they are activated by EDCI and HOBt in presence of catalytic amount of *N*, *N*-dimethylamine pyridine to undergo the nucleophilic substitution in presence of the primary amine in good yield and mild conditions.[5]

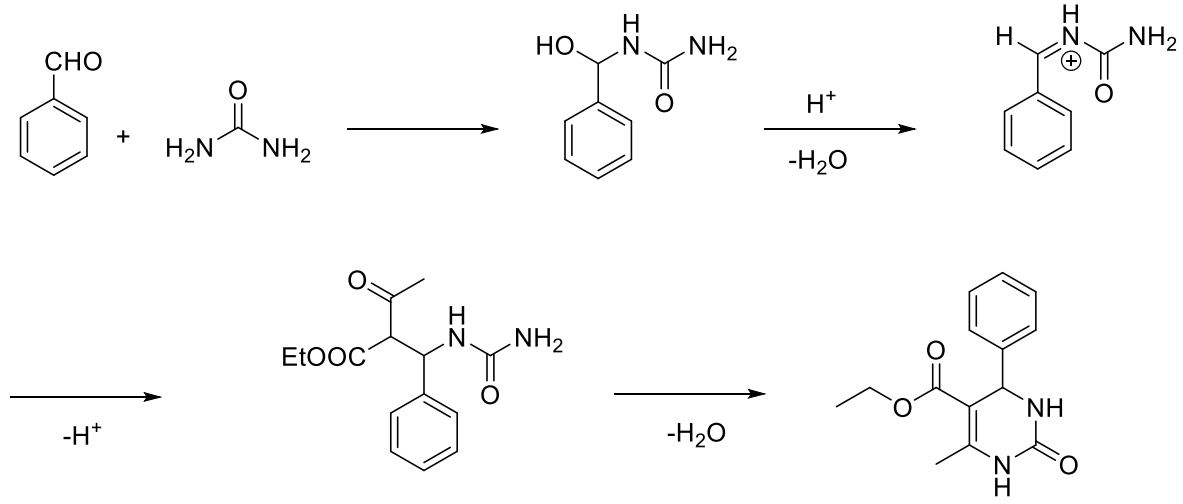
3.2 Second scaffold: analogues of compound 20 (compounds 191-210)

This class of compounds was synthesised using the Biginelli reaction.[12] The reaction of an aldehyde, β-keto ester and urea under acid catalysis in ethanol to obtain a dihydropyrimidine was reported for the first time by Pietro Biginelli in 1893.[13] The classical Biginelli condensation is a multi-component reaction or one-pot condensation reaction, solvent-free and without the use of a catalyst. It was used to generate several compounds with different pharmacological activity such as calcium channel modulator, antihypertensive agents, potassium channel antagonists, anti-HIV agents, antitumor activity (Human kinesis Eg5, (S)-monastrol), antiviral and antibacterial drugs, anti-malarial, anti-epileptics, anti-inflammatory and anti-tubercular compounds.[14] Because of the great value of this class of compounds from a pharmaceutical point of view, over the past 115 years, a lot of different solvents and catalytic conditions were investigated, as well as studies concerning the reaction mechanism leading to the formation of the product and possible intermediates.[16, 15]
The reaction conditions used here are shown in *scheme*[(16)].



Scheme[16]. Reagents *and reagent condition*: **a)** NBS, ethanol, 80° C, 24 hours, yield: 24-92 %.

The general mechanism is shown in *scheme*[17]: it involves first the condensation between the aldehyde and urea, with some similarities to the Mannich condensation reaction, following by the nucleophilic addition of the ketoester enol to the electrophile iminium intermediate. Finally, the ketone carbonyl of the resulting adduct reacts with the urea in the condensation to give the cyclised product.

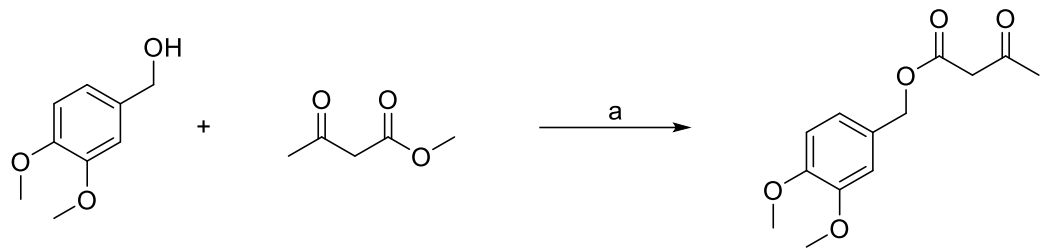


Scheme[17]. Suggested mechanism for Biginelli's reaction.[8, 10]

N-Bromo succinimide (NBS) was used as a catalyst, although its actual role is not clear. Two plausible explanations were postulated, but not yet demonstrated. The former is that NBS may act as a source of Br^+ ions which in turns activates the aldehyde for further reaction with ethyl acetoacetate. The latter is that NBS generates small quantities of HBr or Br_2 which may be the actual catalyst in this reaction and which it seems to be more plausible because the generation of HBr in a protic solvent, such as aqueous ethanol, is more likely and causes the conditions to became slightly acidic.[17]

The synthesis of β-keto esters may occur according to different procedures: mixed Claisen condensation, trans esterification reaction or pyrolysis of 2, 2, 6-trimethyl-4H-1, 3-dioxin-4-one in

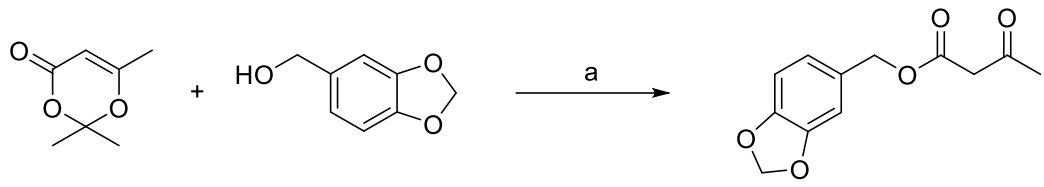
presence of nucleophiles. The preparation of the 3,4-dimethoxybenzyl 3-oxobutanoate intermediate **305** involves a transesterification reaction solvent-free and catalyst-free, heating to 110° C, as reported in *scheme*[(18)].



(305)

Scheme[(18)]. *Reaction condition*: **a)** 110° C, 48 hours, yield: 48 %.

Preparation of β-keto esters is a challenging reaction: the classical and straightforward synthetic route involves either the trans-esterification of ethyl or methyl acetoacetate or treatment of alcohols with aceto-acetylating agents, as aforementioned. Different modifications of this procedure are available, but current limitations are that these reactions are not reproducible and do not afford high yields. Compound **306** was obtained by reaction of the 2,2,6-trimethyl-4H-1,3-dioxin-4-one with benzo[d][1,3]dioxol-5-ylmethanol in presence of potassium acetoacetate at 130° C (*scheme*[(19)]).[18]

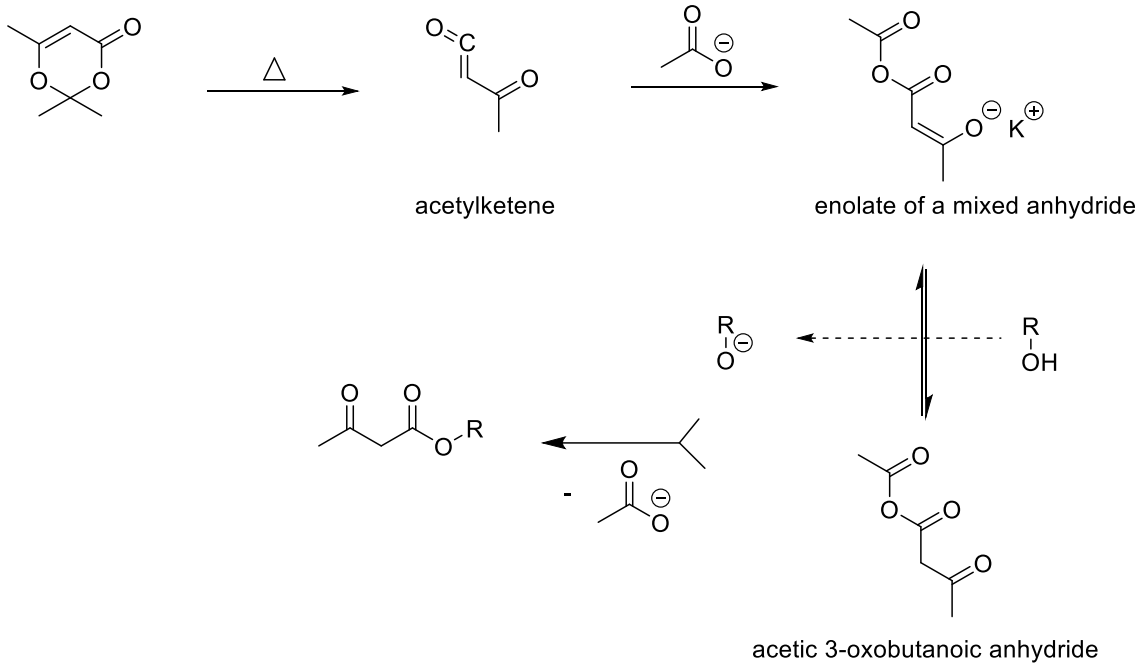


(306)

Scheme[(19)]. Reagents and reagent condition: **a)** potassium acetate, 130° C, 48 hours, yield: 90 %.

Hence, as reported in *scheme*[(20)], the reaction for the preparation of the benzo[d][1,3]dioxol-5-ylmethyl 3-oxobutanoate (**306**) involves thermal degradation or pyrolysis of the 2,2,6-trimethyl-4H-1,3-dioxi-*N*-4-one: potassium acetate generates acetate ions which trap the acetylketene forming enolate of a mixed anhydride. This species participates in a deprotonation equilibrium with alcohol to give the alkoxide and acetic 3-oxobutanoic anhydride, which finally react together through the more electrophilic carbonyl group to furnish the desired β-keto-ester in good yield, higher

reproducibility and mild conditions. The proposed mechanism of reaction is reported in *scheme*[(20)].



Scheme[(20)]. Suggested reaction mechanism.[18]

3.3 Third scaffold: analogues of compound 26 (compounds 211-218)

Analogues of compound **26** (**211**-**218**) have the general structure reported in Figure 47.a. Different modifications of the scaffold were investigated: removal of the benzoic acid and synthesis of asymmetric analogues were explored, as reported in Figure 47.b and c.

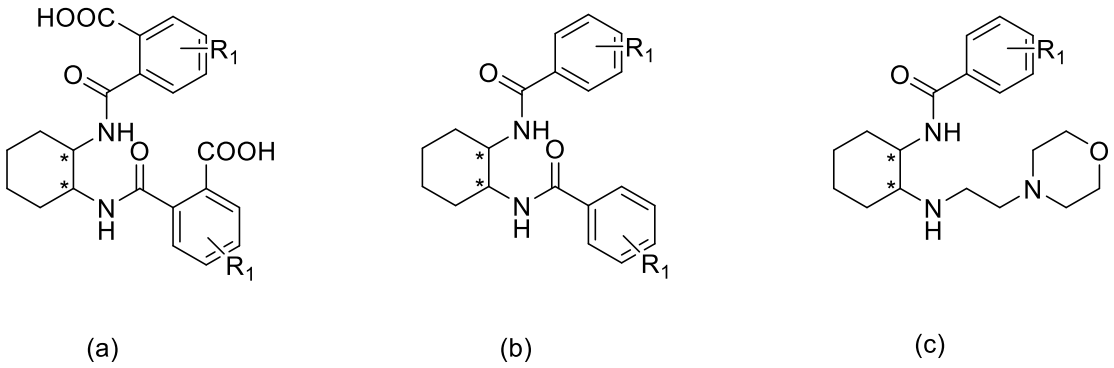
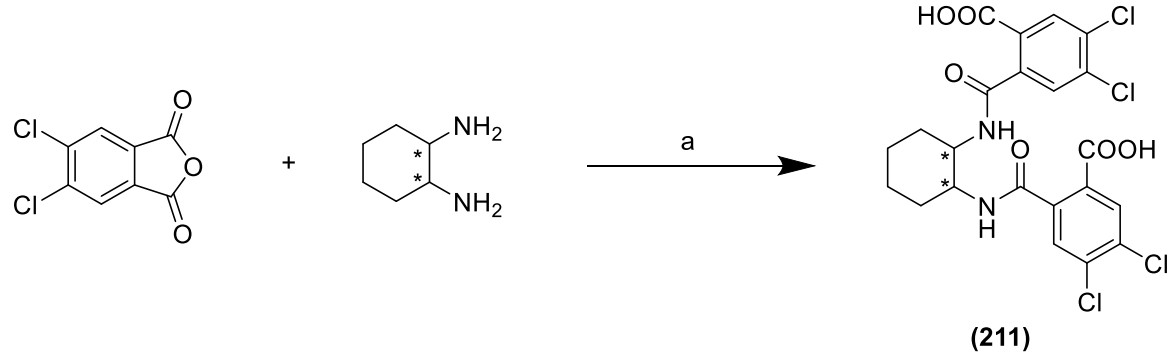


Figure 47. General structure of potential new inhibitors (analogues of compound **26**).

Compound **211** was prepared by reaction of 1, 2-diaminecyclohexyl (mixture of isomers) with

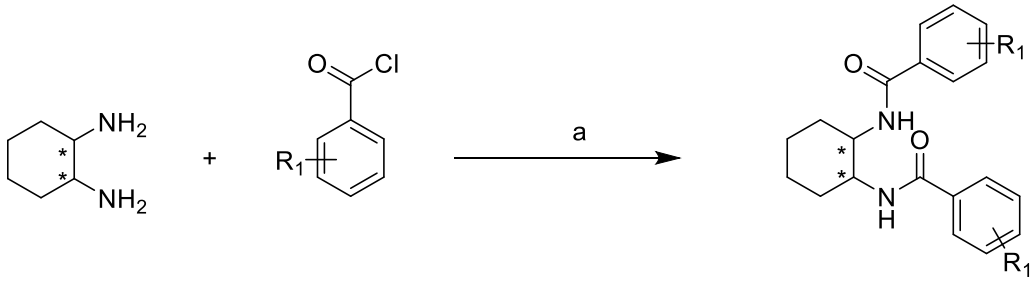
isobenzofuran-1,3-dione, in presence of triethylamine, as reported in *scheme*[(21)].[20] After precipitation of the product, only one species was obtained.



(211)

Scheme[(21)]. **a)** Triethylamine, THF, 25 °C, 12-18 hours, yield: 35%.

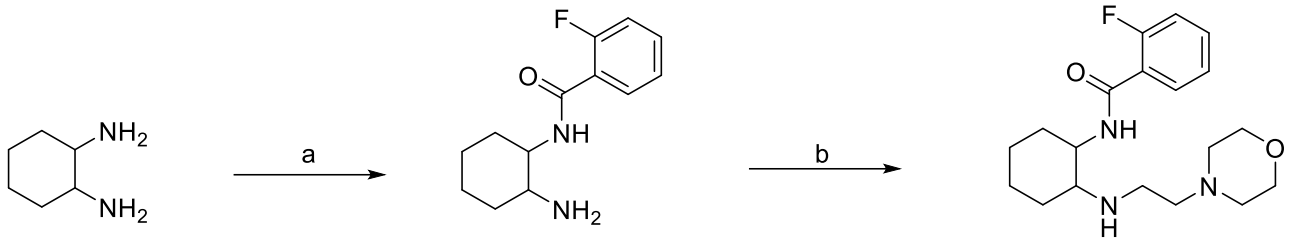
Compounds **212-217** were synthesised by a Schotten-Baumann reaction[21] between benzoyl chloride and cyclohexane-1, 2-diamine in presence of sodium hydroxide to obtain the *N*, *N*'-(cyclohexane-1,2-diyl)dibenzamide, as reported in *scheme*[(22)].



(212 - 217)

Scheme[(22)]. *Reagents and reagent condition:* **a)** sodium hydroxide, 25° C, 15-90 minutes, yield: 56-88 %.

Compound **218** was prepared in a two-step synthetic route, as shown in *scheme*[(23)]: mono-acylation of a symmetrical diamine in presence of 9-borabicyclo [3.3.1] nonane (9-BBN) to provide intermediate **307** and alkylation of a secondary amine (**307)** with the proper alkyl chloride in presence of potassium carbonate in *N*,*N*-dimethylformamide.[22]



(307) **(218)**

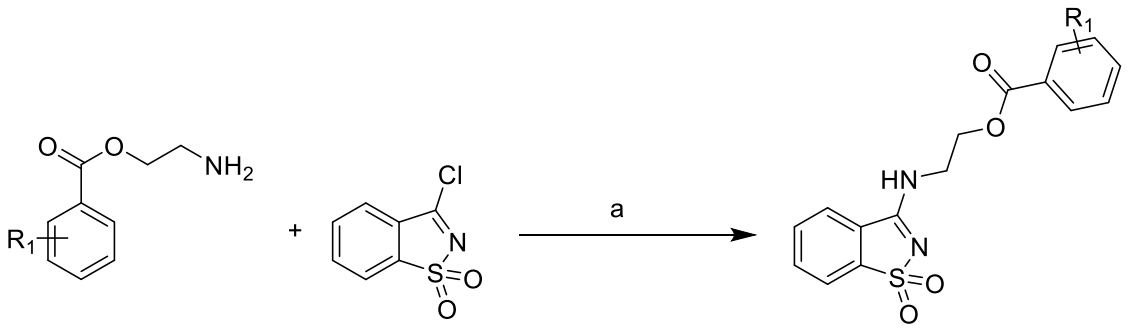
Scheme[23]. Reagents and reagent condition: ***a)*** 9-BBN, 2h, 25° C, yield: 56 %; **b)** 1-chloro-2ethyl morpholine, K_2CO_3, DMF, 80 °C, 1h, yield: 31 %.

The organoborane reagent 9-BBN allows selective complexation of only one of the two nitrogen of diamine with 9-BBN leaves the second nitrogen free to react with the acylating reagents. The aqueous work up decomposes the mono-acylated-mono-9BBN complex, furnishing the titled mono-acylated product with the second nitrogen again free.

The alkylation of primary amine allows formation of secondary amine in good yields.[23]

3.4 Fourth scaffold: analogues of compound 27 (219-221)

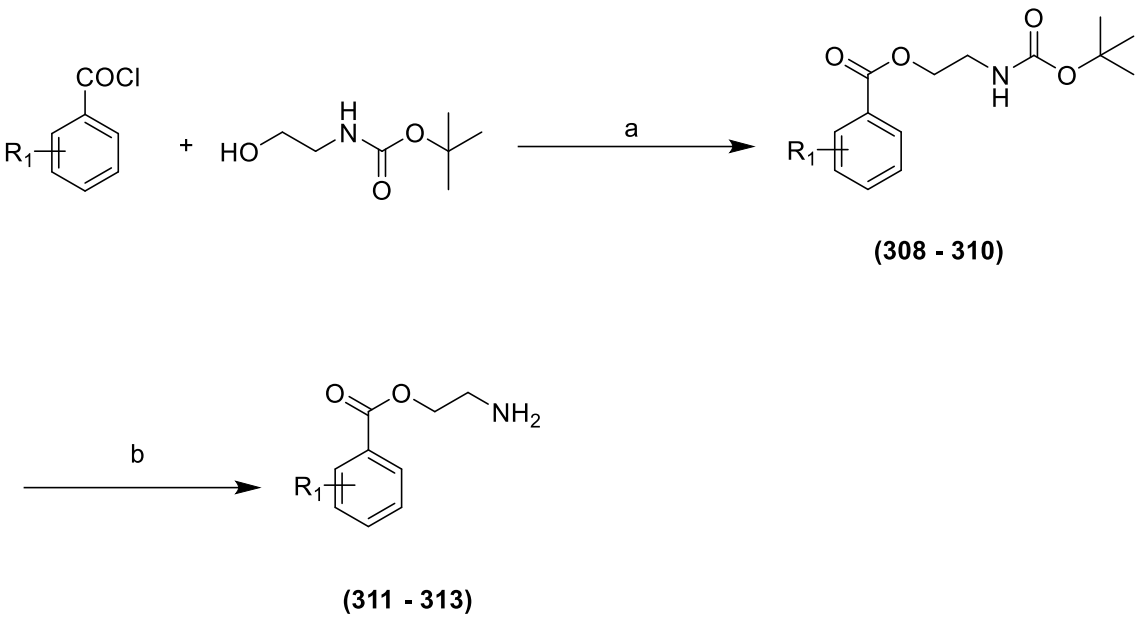
The synthetic route for this class of compounds involve the reaction between the 2-aminoethyl benzoate (compound **311** – **313**) and the 3-chlorobenzo[d]isothiazole 1,1-dioxide (compound **314**) in basic environment, using an aprotic solvent such us tetrahydrofuran, as reported in *scheme[24]*.



(311 - 313) **(314)** **(219 - 221)**

Scheme[24]. Reagents and reagent condition: **a)** triethylamine (5 equivalents), 25° C, 1-16 hours, yield: 14-25 %.

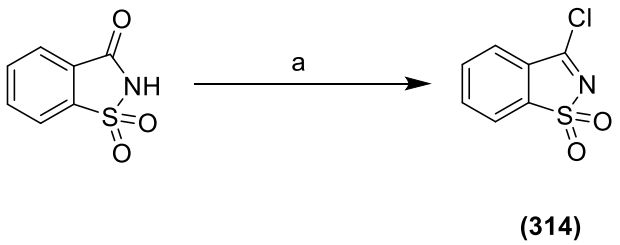
As reported in *scheme[25]*, the β-amino ester is prepared in a two step synthetic route starting with the *N*-tert-butyloxycarbonyl protected ethanolamine to avoid formation of *N*-(2-hydroxyethyl)benzamide as by-product.



Scheme[25]. Reagents and reagent condition: **a)** sodium hydroxide 10%, 25° C, 1-4 hours, yield: 65-76 %; **b)** trifluoroacetic acid, dichloromethane, 40 °C, 1 – 3 hours, yield: 33-71 %.

Deprotection was performed in presence of an excess of trifluoroacetic acid (TFA) either at room temperature or heating at 40 °C.

3-Chlorobenzo[d]isothiazole 1,1-dioxide (**314**) was obtained by chlorination of benzo[d]isothiazol-3(2H)-one 1,1-dioxide in presence of thionyl chloride using 1, 4-dioxane as solvent and catalytic drops of *N*,*N*-dimethylformamide (*scheme [26]*). Different chlorinating agents were investigated, but neither phosphoryl chloride nor phosphorus pentachloride led to the conversion of the benzo[d]isothiazol-3(2H)-one 1,1-dioxide in 3-chlorobenzo[d]isothiazole 1,1-dioxide (**314**) in high yield, conversely achieved with thionyl chloride.



Scheme [26]. Reagents and reagent condition: **a)** thionyl chloride, 110° C, 48 hours, yield: 70 %; or **a)** phosphoryl chloride, 170 °C, 24 hours, yield: 52%; or **a)** phosphorus pentachloride, 180 °C, 6 hours, yield: 41%.

The reaction provided the desired product (monitored by the shift in the ^{13}C-NMR of carbon in 3

position) in high yield.

3.5 Analogues of compound 23 (compounds 222-225): quinazolinone

Formation of an intra-molecular hydrogen bond between the amide moieties on the original scaffold, observed during the computational studies, resulted in conformational restriction by virtual ring. Hence, a further modification on compound **23** is the introduction of a closed central ring. Two possibilities were explored: quinazolinone based scaffold as shown in Figure 48 and isoquinoline based scaffold (section 3.6).

Docking studies demonstrate similarity of binding pose of this new scaffold with compound **23**. As shown in Figure 48.b, morpholine ring is orientated towards the new small binding cavity, while the 2-fluoro phenyl ring is oriented down toward a lipophilic part of the bigger binding pocket. Compound **23** is stabilised by hydrogen bond interaction with Ser807, whereas compound **225** is anchored by hydrogen bond interaction with Arg811.

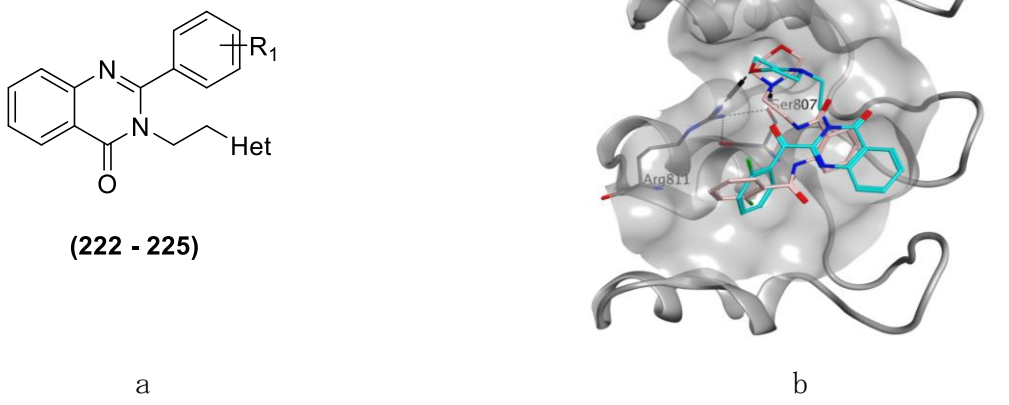
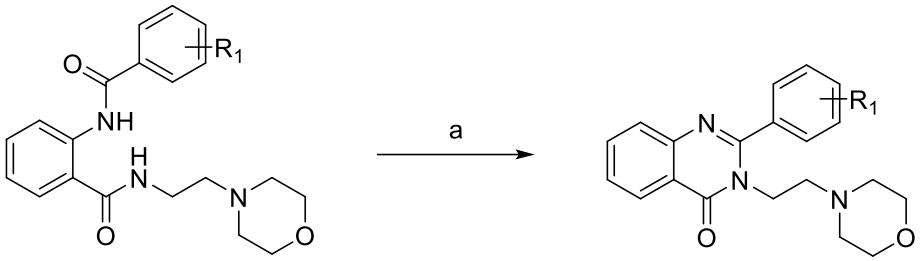


Figure 48. **a)** General structure of compounds **222** – **225**. **b)** Alignment of compound **225** (light blue) with **23** (pink).

The synthetic pathway, reported in *scheme*[27], allows closure of the ring in presence of formamide: the reaction was performed first with the conventional heating at 200 °C for 24 hours, with low yield. Hence, the microwave irradiation at 210 °C, 200 W, over shorter period of time (10 – 45 minutes) successfully furnished the desired products in really good yields.[7]

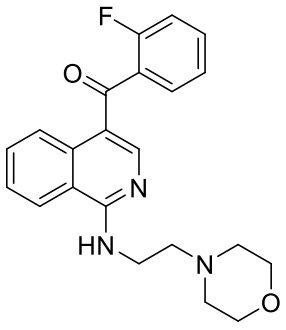


(23, 31, 86, 186) **(222, 223, 224, 225)**

Scheme[27]*. Reagents and reagent condition:* **a)** formamide, 210 °C, 10-45 minutes, yield: 36-63%.

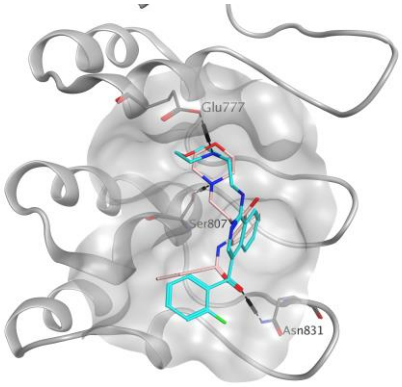
3.6 Analogues of compound 23 (compound 226): isoquinoline

Figure 49 shows another possibility to close the central ring. The isoquinoline based scaffold is characterised by a central isoquinoline core, substituted in position 1 with 2-morpholinoethan-1-amine and in position 4 with different benzophenones (Figure 49.a). As reported in Figure 49.b, isoquinoline central core is stabilised in a lipophilic part of the main binding pocket, fitting the morpholine ring toward an upper and smaller binding cavity: in this pose, compound **226** is anchored by two hydrogen bonds with key amino acid residues (Asn831 and Glu777) in a more stable pose. Conversely, compound **23** is kept in the binding pocket only by one hydrogen bond with Ser807.



(**226**)

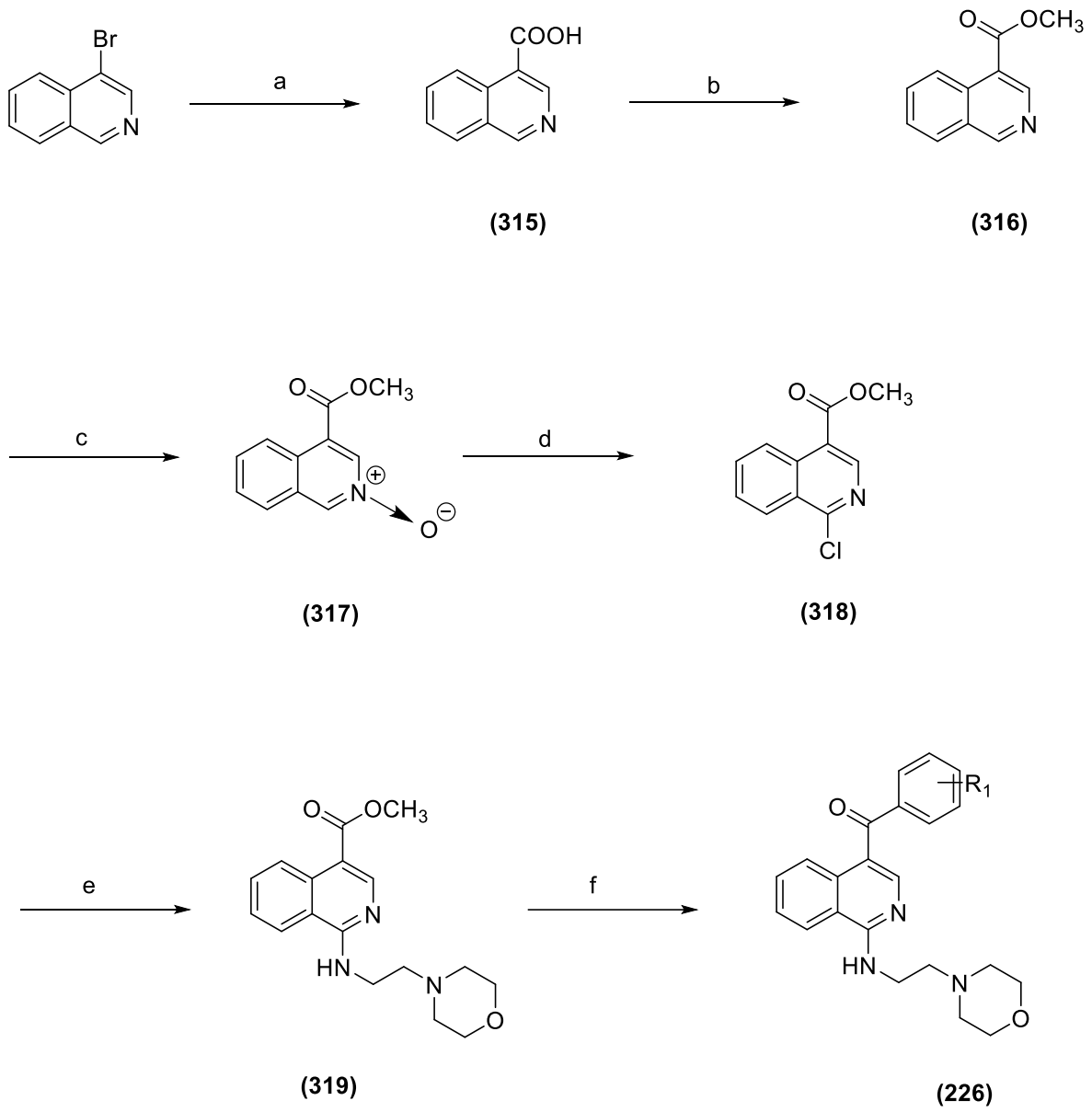
a



b

Figure 49. a) Structure of compound **226**. b) Alignment of compound **226** (light blue) with compound **23** (pink).

The synthetic strategy provides for a seven-steps synthetic route as shown in *scheme*[28].



Scheme[28] *Reagents and reagent condition:* **a**) t-BuLi 1.9M in pentane, carbon dioxide, THF, -80 °C, 1 hour, yield: 68%; **b**) EDCI, HOBt, DMAP, CH_3OH, CH_2Cl_2, 16 hours, 25 °C, yield: 72%; **c**) mCPBA, dichloromethane, 14 hours, 0-25 °C, yield: 84%; **d)** $POCl_3$, DMF, CH_2Cl_2, 160° C, 8 hours, yield: 85 %;EDCI, HOBt, DMAP, CH_3OH, CH_2Cl_2, 16 hours, 25 °C, yield: 71%; **e)** 2-morpholinoethan-1-amine, pyridine, 100 °C (microwave irradiation), 80 W, 10 minutes, yield: 70%; **f)** CF_3SO_3H, toluene, F-Ph, 85°C.[13]

In the first step, lithiation of bromide and quenching with dry ice furnish the carboxylic acid (compound **315**), which is then protected with the methyl ester (compound **316**), using the activated ester methodology due to high yield and short reaction time.

Successively, oxidation of the nitrogen atom in presence of meta-chloroperoxybenzoic acid to give the *N*-oxide derivative (compound **317**) is necessary to force the next step of the synthetic route to selectively perform the chlorination at C1 position using phosphoryl chloride (compound **318**). In step *e*, nucleophilic substitution on aromatic carbon (C1) was performed according to two different

strategies: initially, the Buckwald-Hartwig amination of aryl halides (in presence of BINAP, palladium acetate and cesium carbonate in toluene)[8, 9] was performed, but only traces of the product were obtained because of insolubility of the starting material in the solvent. Hence, a more efficient approach was used: by means of microwave irradiation, it was possible to obtain the desired methyl 1-((2-morpholinoethyl)amino)isoquinoline-4-carboxylate (compound **319**) in good yield, over a shorter reaction time and without the use of costly palladium catalyst or harsh conditions. On the ester (compound **319**) a new procedure to generate aryl ketone was applied, as reported in the literature.[10] The use of superacids, such as trifluoromethansulfonic acid, in order to super-electrophilically activate the eletrophiles does not afford the isolation of the desired product. Hence, a new strategy was applied: hydrolysis of the ester leads to the formation of the free carboxylic acid. The Suzuki coupling of boronic acid with carboxylic acid was performed in presence of tetrakis(triphenylphosphine)palladium(0), tripotassium phosphate and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) to *in situ* activate the carboxylic acid. Two different solvent systems were explored: 1,4 dioxane, and a mixture of toluene/ethanol/water. The starting materials were not soluble in both solvent systems, hence this may explain the unsuccessful synthesis of the desired compound. Moreover, on the isoquinoline-4-carboxylic acid (compound **315**), two different Friedel-Crafts acylation were performed: in the first case, the acid chloride was prepared with thionyl chloride and catalytic DMF, while in the second case with the oxalyl chloride and catalytic DMF. Also in this case, it was not possible to isolate the desired product. On compound **315** also the Suzuki coupling was performed again with the same conditions: but only traces of the final product were detected by mass spectrometry.

As a result, different solvent system will be used to improve the Suzuki coupling reaction, but also different catalyst. Moreover, the acylation with the superacids will be performed not on the intermediate (compound **315)** with the morpholine, but on the one with the chlorine atom in 1 position. Moreover, Friedel-Crafts acylation will be conducted with new reagents to ensure that the formation of the acid chloride was not hampered by the presence of old reagents.

3.7 Functionalised analogues of compound 23

Compounds **227** – **229** were synthesised in order to be used by Nanotethers Discovery Science Limited company. Nanotethers technology is a new technology from Nanotether Discovery Science (NDS) to explore protein-protein interactions or small-molecule/target protein interaction. Nanotether is a new tool, which may be used to demonstrate the on-target action of drugs. Briefly,

recombinant protein (Bcl-3) and chemical probe (compounds **229**-**231**) are attached to the end of a nanoscale tether and their binding may be quantified by fluorescence resonance energy transfer (FRET), as shown in Figure 50.

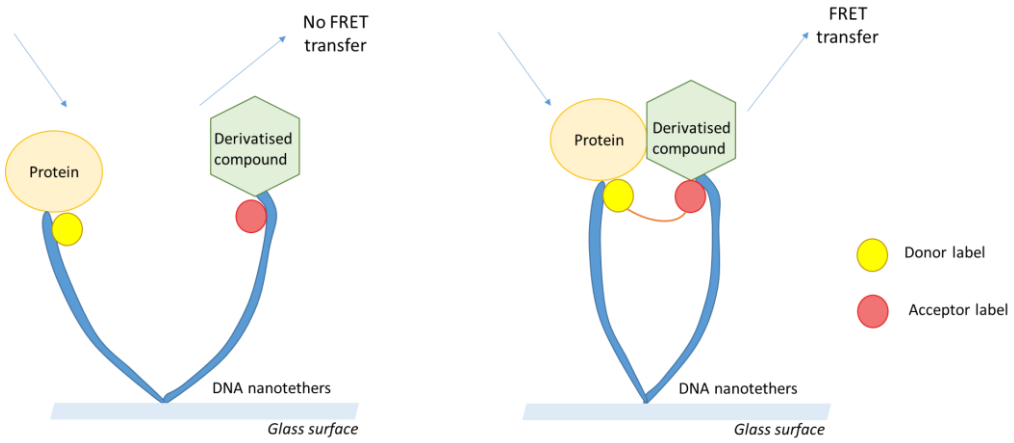
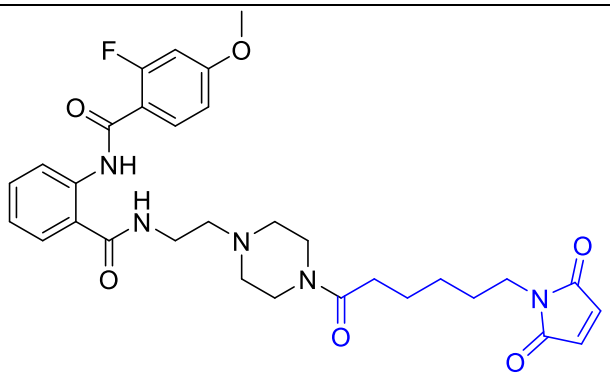


Figure 50. Nanotethers technology system: **a)** unbound state; **b)** bound state.

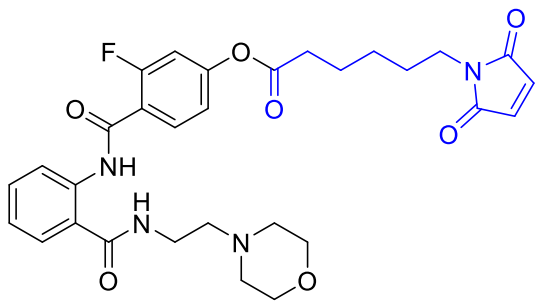
In the unbound state, the modified compound does not dock the binding site on Bcl-3 protein, thus the laser pulse is absorbed by the donor label, but not transferred to the acceptor. Conversely, in the bound state, the functionalised compound docks into Bcl-3 binding site and pulse light absorbed by donor is transferred to the acceptor and re-emitted. The herein presented strategy will be used to demonstrate that **23** and **31** (section 5.2 for further details on their biological activity) are potential inhibitors for the designed target.

The tethers are composed of thiolated oligonucleotide. Three chemical probes were synthesised, as derivatives of compound **23** and **31**. The Michael acceptor 7-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) heptanoic acid group was introduced to alkylate thiolated oligonucleotide and to attach the test compounds to the oligonucleotide.

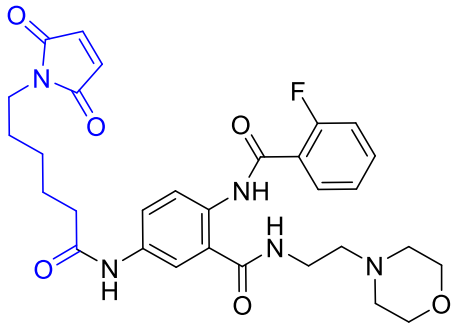
Three different insertion points were designed and successfully synthesised, as reported in Figure 51. Purifications of the final compounds were performed directly by Nanotethers collaborators.



Compound **227**



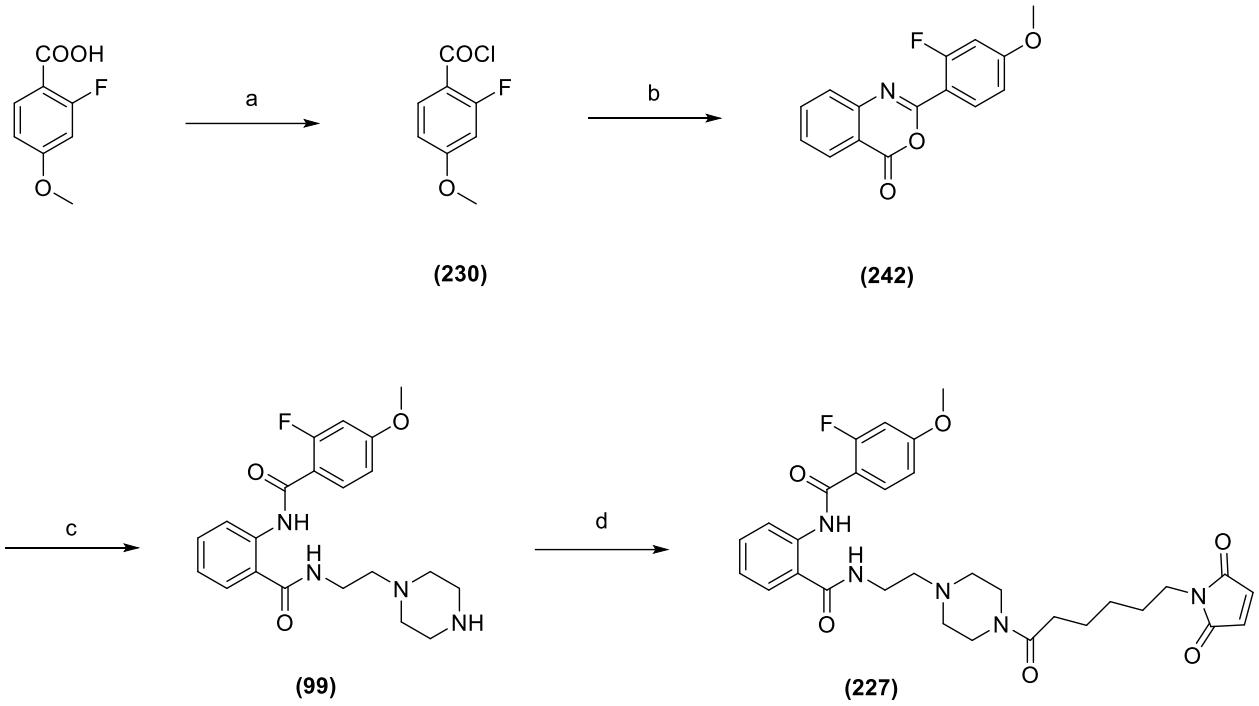
Compound **228**



Compound **229**

Figure 51. General structure of functionalised derivatives.

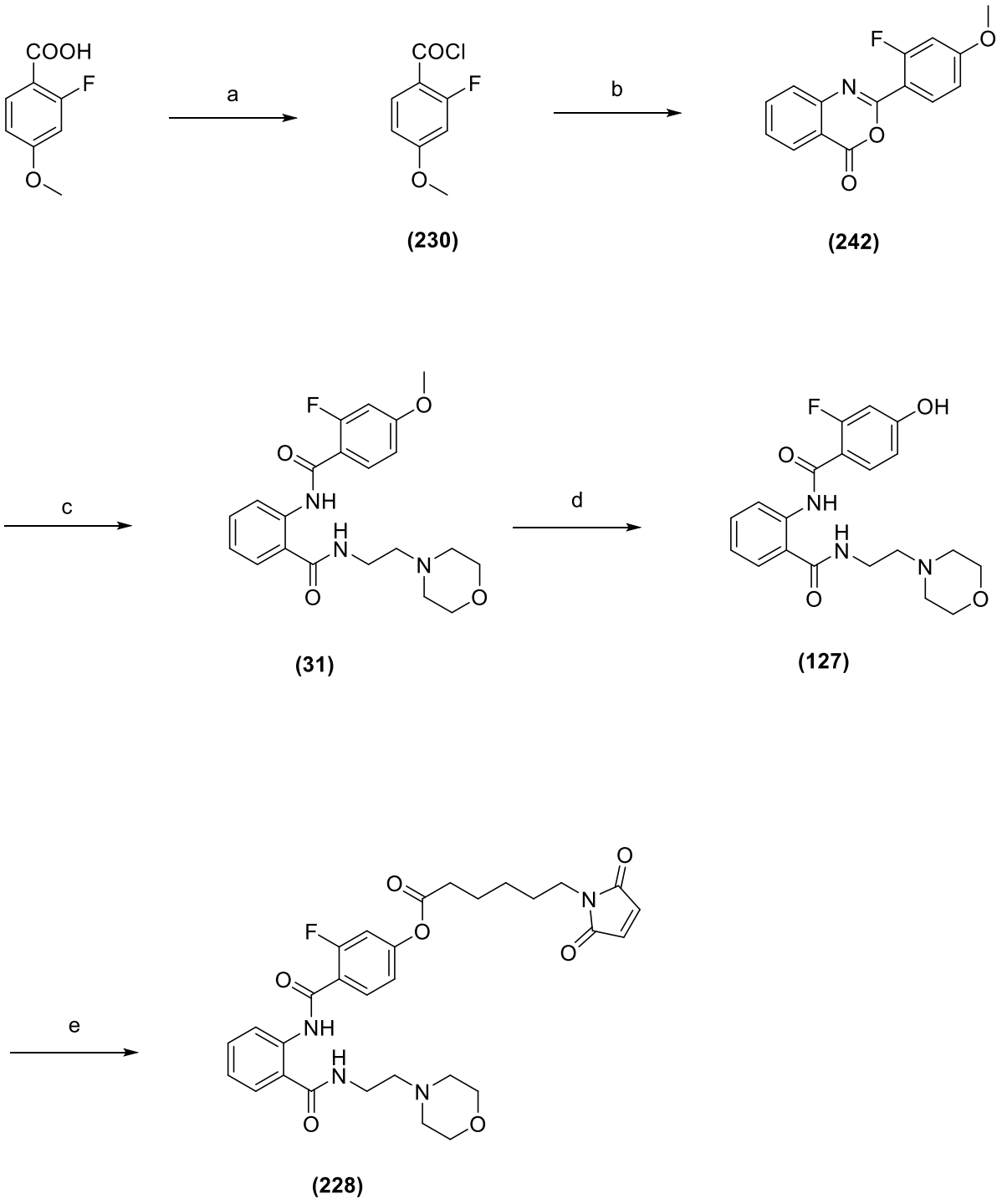
Compound **227** was synthesised according to the original synthetic route as previously described: conversion of carboxylic acid into more reactive acid chloride (step a, *scheme*[29]) was achieved as previously discussed in *scheme*[10]. Nucleophilic acid substitutions and cyclo condensation of benzoyl chloride and anthranilic acid chloride (step b → c, *scheme*[29]) were performed as already reported in *scheme*[1] and *scheme*[3]. In the last step of reaction, *in situ* activation of 7-(2, 5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)heptanoic acid with TBTU affords coupling with secondary amine on piperazine ring of compound **99**. The crude product (**227**) was directly used to perform the final coupling with cysteine residues by Nanothethers, without further purification.



Scheme[29]. Reagents and reagent condition: **a)** $SOCl_2$, DMF, 80 °C, 6 hours; **b)** anthranilic acid, pyridine, 25 °C, 5 hours, yield: 45%; **c)** 4-(2-Aminoethyl)morpholine, DMF, DIPEA, 25°C, 16 hours; yield: 33%; **d)** 6-Maleimidohexanoic acid, TBTU, DIPEA, DMF, 25°C, 16 hours.

As reported in *scheme[30]*, synthesis of compound **228** is characterised by 5 steps synthetic route. Steps (a-d) follow the usual synthetic route for the first scaffold of analogues to yield compound **31**, as previously described in section 3.1: conversion of carboxylic acid into more reactive acid chloride (step a, *scheme[30]*) was achieved as previously discussed in *scheme[10]*. Nucleophilic acid substitutions and cyclo-condensation of benzoyl chloride and anthranilic acid (step b → c, *scheme[30]*) were performed as already discussed in *scheme[1]* and *scheme[3]*. Upon demethylation reaction in presence of boron tribromide,[3] the hydroxyl group in 4 position is now ready to react with the 7-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)heptanoic acid, previously activated *in situ* with HOBt yielding the final product.[5]

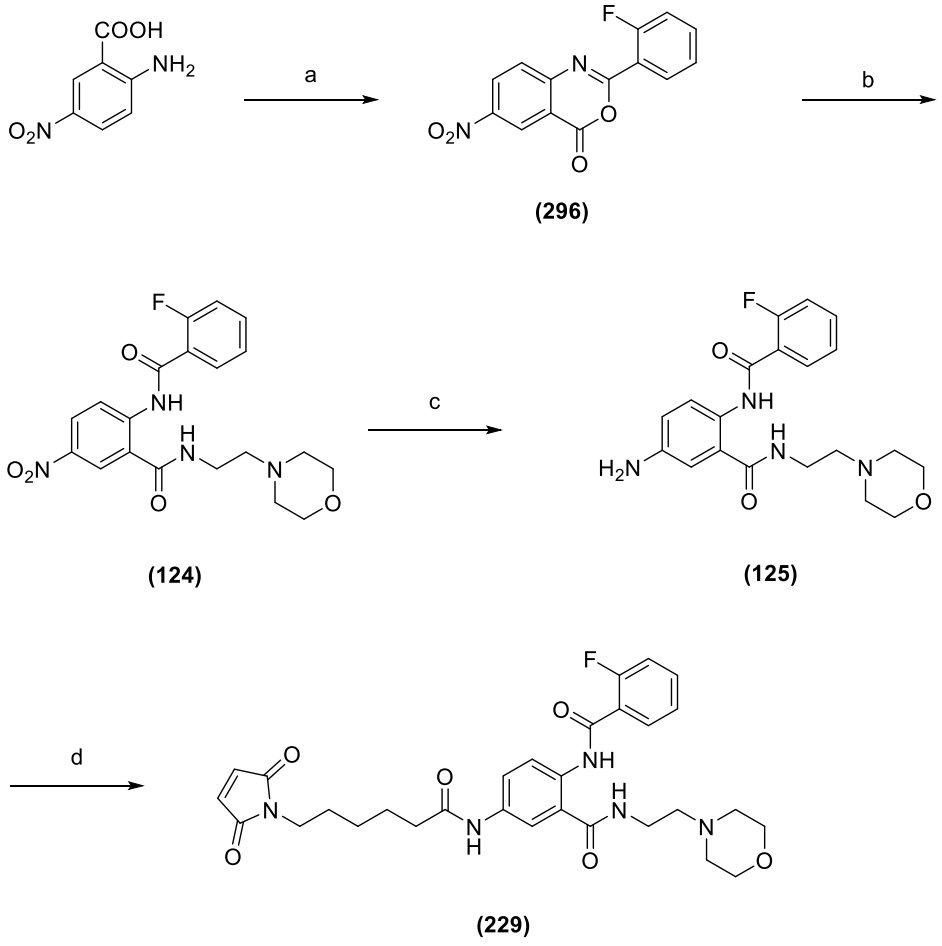
Crude compound was sent to Nanotethers without further purification.



Scheme[30]. Reagents and reagent condition: **a)** $SOCl_2$, DMF, 80 °C , 6 hours; **b)** anthranilic acid, pyridine, 25 °C, 5 hours, yield: 45% **c)** 4-(2-Aminoethyl)morpholine, DMF, DIPEA, 25°C, 16 hours; yield: 57%, **d)** BBr_3, dichloromethane, -78 °C, 24 hours; yield: 38%, **e)** 6-Maleimidohexanoic acid, EDCI, HOBt, dichloromethane, 25°C, 16 hours.

Synthesis of compound **229** involves a 4 step synthetic route, as shown in *scheme[31]*: compound **124** was synthesised according to the usual synthetic route, as previously described in section 3.1. Steps (a-c) follow usual synthetic route for the first scaffold of analogues, as previously described in

section 3.1. Nucleophilic acyl substitutions and cyclo-condensation of benzoyl chloride and anthranilic acid chloride (step b → c, *scheme*[(31)]) were performed as already discussed in *scheme*[(1)] and *scheme*[(3)]. Due to solubility limitations, hydrogenation reduction in presence of palladium on carbon provided the reducted amino compound **125** only in low yield (8%). Thus, reduction of the nitro group in presence of tin (II) chloride dihydrate in ethyl acetate[25] was used and provided the desired aromatic amine in good yield (59%) which underwent cross-coupling with activated ester 1H-benzo[d][1,2,3]triazol-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) hexanoate to provide the desired compound **229**.



Scheme[(31)]*. Reagents and reagent condition:* **a)** 2-fluorobenzoylchloride, pyridine, 25 °C, 5 hours, yield: 85%; ***b)*** 4-(2-Aminoethyl)morpholine, DMF, DIPEA, 25°C, 16 hours; yield: 34%; **c)** $SnCl_2$, AcOEt, 85°C, 10 hours; yield: 59%; **d)** 6-Maleimidohexanoic acid, EDCI, HOBt, dichloromethane, 25°C, 24 hours.

3.8 Further characterization of compounds 31: limit of detection and limit of quantification

Compound **31** is under evaluation for several pharmacokinetic parameters: it was necessary calculate the limit of detection (LoD) and the limit of quantification (LoQ). The spectrophotometric measurements were carried out using a Cary 300 UV-Vis-NIR spectrophotometers with 1 cm matched quartz cell. Cary WinUV software was used to process data.

Preparation of standard solution

Compound **31** (1 mg; 0.00249 mmol) was dissolved in 10 mL of methanol. The concentration of the stock solution was 0.00025 M: it was scanned in UV range (200-400 nm) in 1.0 cm cell against solvent as blank; the spectrum was obtained.

Determination of λ_{max}

1 mg/ 10 mL of compound **31** was prepared and scanned in UV range (200-400 nm). The λ_{max} was found to be at 272 nm wavelength. Absorbance is 1.08 at 272 nm (Figure 52).

Preparation of samples

Standard stock solution was diluted with methanol to obtain a range of 10-fold concentrations: 0.25 mM, 0.025 mM, 0.0025 mM, 0.00025 mM, 0.000025 mM. Blank sample is 0.7 mL of methanol (Figure 53).

Concentration graph was plotted for concentration and absorbance: the equation of calibration curve is: $y = -0.2342x + 1.0784$ and R^2 is 0.83 as shown in Figure 54.

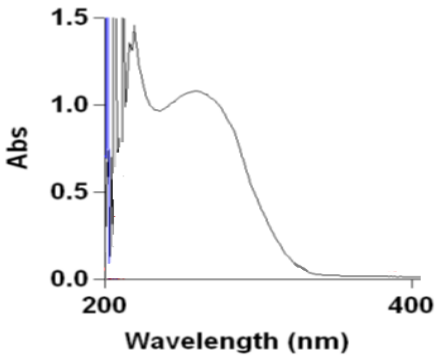


Figure 52. Absorbance at 272 nm, concentration 0.25 mM.

Samples concentration	Absorbance	± SD
2.5 mM	1.036	± 0.022
0.25 mM	0.51	±0.003
25 µM	0.176	±0.001
2.5 µM	0.111	±0.001
0.25 µM	0.059	±0.006
Blank	0.049	± 0.00094

Figure 53. Absorbance at 272 nm, different concentrations. Mean ± SD.

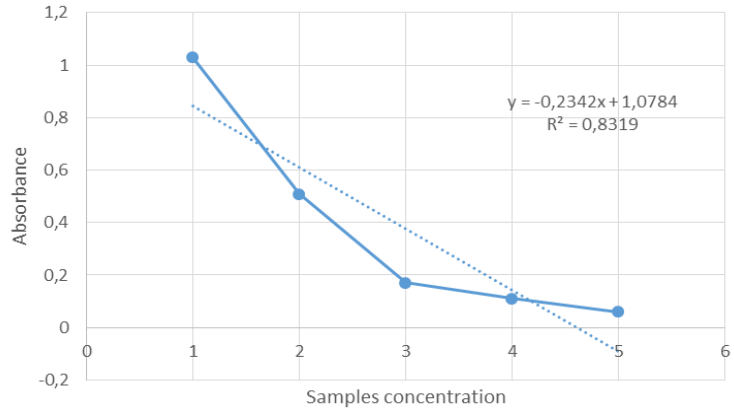


Figure 54. Linear regression and equation.

According to the International Conference of Harmonisation (ICH), the LoD is defined as the "lowest amount of analyte in the sample, which can be detected but not necessarily quantified under stated experimental conditions", while the LoQ is defined as "lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy".[24]

In order to determine the detection and quantification limit, there are different methods that may be used:

1) visual definition;
2) calculation from the signal-to-noise ratio;
3) calculation from the standard deviation of the blank.

When the standard deviation of the blank is a non-zero result, LoD may be determined as:

$LoD = 3.3\ \sigma_{b1} / m$ $LoQ = 10\ \sigma_{b1} / m$

where σ_{b1} = standard deviation of the blank; m = slope of the regression curve

According to the calculations, LoD = 0.0134 mM and LoQ = 0.040 mM.

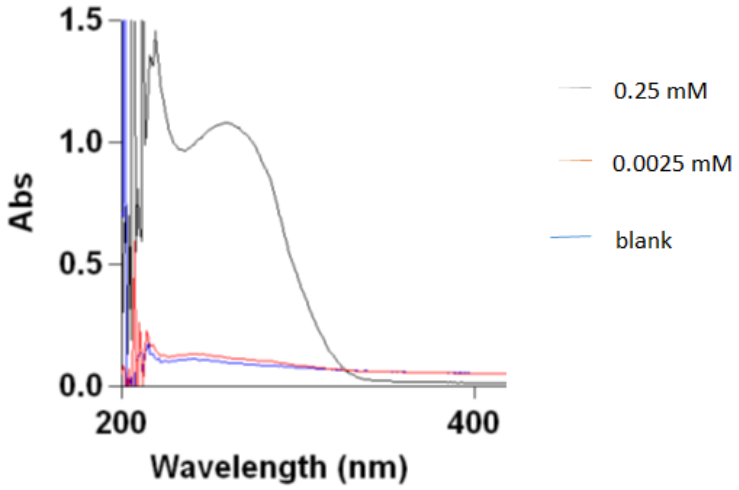
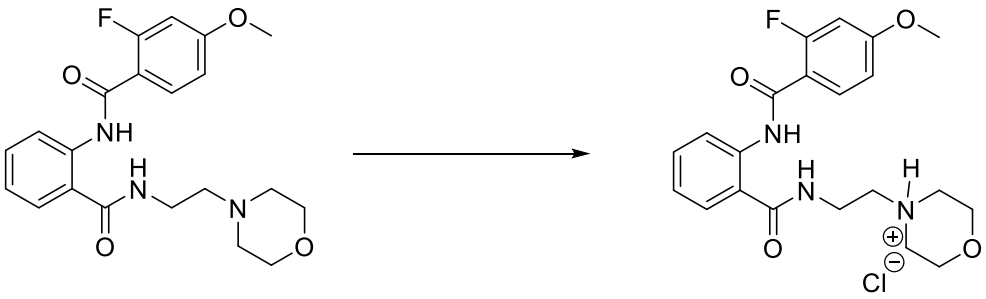


Figure 55. Absorbance of stock solution, sample and blank.

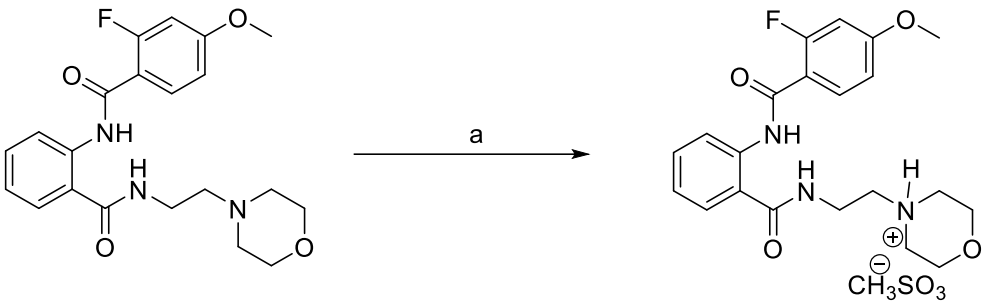
3.9 Preparation of salt of compound 31

To increase solubility of compound **31**, different salt forms were prepared. The preparation of the chloridate salt **31.a** of compound **31** involved dissolution of **31** in methanol and addition of hydrochloric acid 1.25 M solution in methanol.



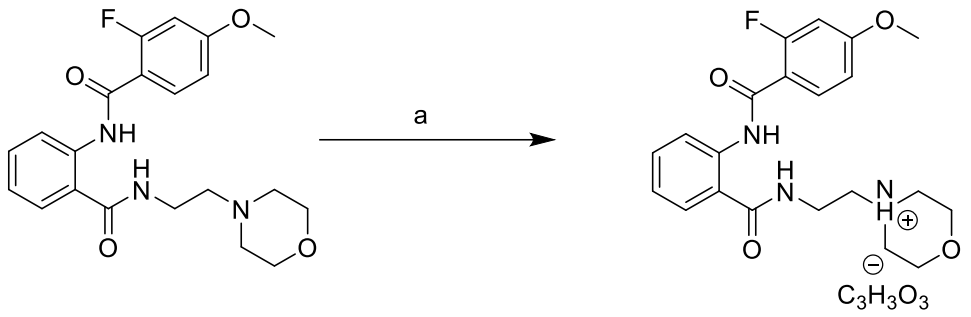
Scheme[32]. Chloridrate salt preparation.

The preparation of mesylate salt **31.b** involved the dilution of compound **31** in dichloromethane; one equivalent of methanesulfonic acid was added. The solvent was evaporated and the salt form collected.



Scheme[33]. Mesylate salt preparation.

To obtain the malonic salt form **31.c**, compound **31** was dissolved in acetone and one equivalent of malonic acid was added. The evaporation of the solvent allowed the recovery of the product as a yellow oil.



Scheme[34]. Malonic salt preparation.

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Section 4:

DESIGN AND DEVELOPMENT OF BIOLOGICAL ASSAYS

4.1 Mycoplasma assay

Cell lines used in bioassays were routinely tested with Mycoplasma test assay (protocol in section 7.4). Mycoplasma is a genus of bacteria characterised by absence of a cell wall, fried-egg-shaped colonies and the ability to pass through 200-nm pore diameter membrane filters. Because of their lack of a cell wall and tiny dimensions, they are not affected by commonly used antibiotics. Thus, Mycoplasma may infect cultured cells even if they are kept in an antibiotic containing medium. It was shown that up to 80% of cell lines may be contaminated by different species of Mycoplasma.[1] As a result, the presence of Mycoplasma may affect the experimental data when a contaminated cell culture is used resulting in erroneous outcomes or causing the loss of unique cell lines. Thus, it is necessary to perform Mycoplasma detection assay to ensure that cell cultures are not contaminated.
To check for the deleterious Mycoplasma contamination, cells are analysed using the PCR Mycoplasma Test Kit 1/C: the kit is based on a PCR method and contains a positive control (sample containing *Mycoplasma* contamination) and a negative control (non-contaminated sample). All cell lines used in the course of this thesis were routinely checked for *Mycoplasma* contamination. As an example, results from one gel are reported in Figure 56.[2]

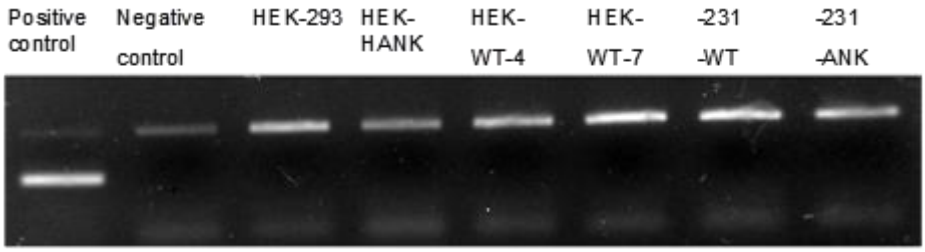


Figure 56. Gel evaluation: results of Mycoplasma assay in HK-293 cell line and its sub-cell lines. Lack of positive control band in the samples demonstrates that the Mycoplasma infection is not present.

4.2 Cell viability assay

A cell viability assay measures the presence of a marker activity, reported to be associated with viable cells. It may be used to illustrate the fate of cells in the presence of tested compounds and thus to investigate the cytotoxic profile of novel drugs.[3] Several techniques are available to measure cell viability, including the MTT Tetrazolium Assay, the Cell Titer Blue assay or the Protease Viability Marker Assay.[4] In this scenario, the Cell Titer Blue assay, termed also resazurin reduction assay, is the only one recommended for high-throughput screening of compounds in 96 -well plates as it exhibits enhanced efficiency because of relatively short end-point and reproducibility across different

cell lines.[5] In contrast, the MTT assay is generally less sensitive than fluorescent methods to detect viable cells: due to this disadvantage, its application in high-throughput screening is limited.[6] Otherwise, the high cost for a protease marker activity based cell assay is an influential parameter considered in the choice of the cell viability assay.

However, the cell titer blue viability assay is a fluorimetric method based on the detection of resazurin, an indicator of cell viability: it measures the metabolic capacity of viable cells to reduce resazurin, as depicted in Figure 57.[3]

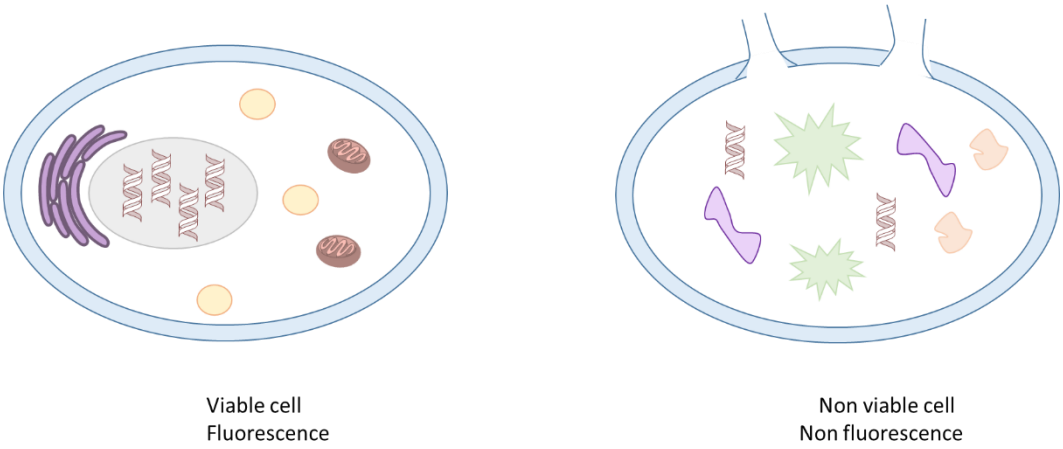
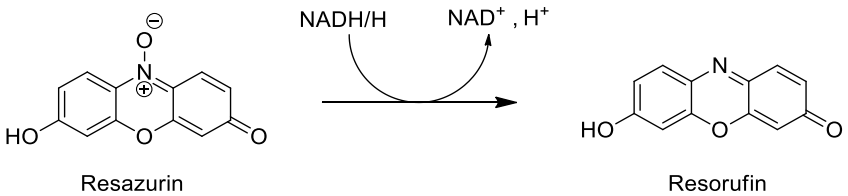


Figure 57. Fluorescence in viable and non viable cells.

Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is a blue dye, weakly fluorescent. Upon reduction in mitochondria, it emits a strongly blue fluorescent signal as resorufin.[3] The Cell Titer-Blue® Reagent (made up of highly purified resazurin, Promega) undergoes a "blue shift" upon reduction of resazurin to resorufin: resazurin has a maximum absorbance at 605 nm, while maximum absorbance for resorufin lies at 573 nm. In the reduction-oxidation mechanism, while resazurin is reduced to resorufin, the NADH is oxidized to NAD^+ in presence of NADPH dehydrogenase or NADH dehydrogenase, [5] as shown in *scheme* [(35)].



Scheme[(35)]. Reduction of resazurin to resorufin in living cells.

Thus, viable cells keep the ability to reduce resazurin into resorufin. Conversely, non-viable cells lose metabolic capacity, do not reduce the indicator dye and so they do not generate a fluorescent signal. The number of viable cells is proportional to the quantity of produced resorufin, which may be detected by absorbance and fluorescence. Positive and negative controls may be used to define the appropriate assay window. The positive control is a compound known to be toxic to the investigated cells (G418A and Carboplatin). The negative control (Dimethyl sulfoxide) is a chemical compound, which is known not to be toxic to the investigated cells and is used in equimolar concentrations as vehicle for the dilutions of tested compounds. Normalising the raw data to the negative control then allows the characterisation of the cytotoxic profile of tested compounds without effect due to putative proliferative effect of the vehicle, DMSO, on the cell lines.[7]

4.2.1 Optimisation of the Cell Titer Blue assay

Initially, general conditions according to manufacturer's instructions were used to perform cell viability assay on a highly metastatic cell line (MDA-MB-231, cell line further details in section 7.1, general protocol in section 7.7). However, due to variability within the same plate and also among different plates of both controls and the reference compounds **23** and **186**, an optimisation of the general protocol was necessary. The results when using the general protocol for compounds **23**, **186** and the positive control are reported in Figure 58: the magnitude of the observed variability for each of the five independent repetitions demonstrates that this protocol is not reproducible for the investigated cells and thus needed to be optimised.

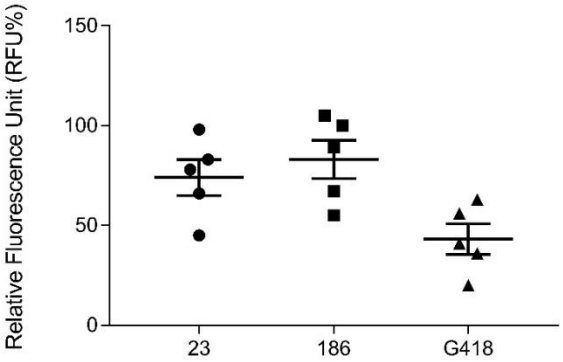


Figure 58. Results of the cell titer blue assay before optimisation of the general procedure for compounds **23**, **186** and G418 as positive control. Cells were seeded at 3000 cells/well and cultured at 37 °C, 5% CO_2 for 24 hours. Cell titer blue reagent was incubated for one hour and absorbance was measured on the luminescence microplate reader, Clariostar (BMG Labtech). Individual measurements are normalised against negative control. Mean ± SEM, N=5, n=3.

To achieve reproducible and reliable results, non-ideal parameters in the general protocol were identified and optimised, as presented in Table 7.

Table 7. Summary of parameters modified in the optimisation process for cell viability assay.

	Parameter	Problem	Solution
Biochemical problems	number of cells seeded	too low (3000 cells/well)	100000 cells/well
	incubation time	1 hour	2 hour
	positive control	G418, Neomycin	Carboplatin
Mechanical problems	counting cells	hematocyter non accurate	automated cell counting
	plate layout	variability across plate and plates	different layaout

Generally, a low sample size negatively affects the accuracy of biological results: in this case, 3000 cells per well seemed to be a too sparse cell density rate for a 96-well plate. Increasing the sample size, cell number and therefore the signal strength statistically improved the reliability of results by decreasing the errors of the individual observations.

Each cell line requires a different incubation time with cell titer blue reagent to reduct resozurin into resorufin. A 1 hour incubation time proved to be a too short period of time for MDA-MB-231 cells to effectively perform the reduction. Thus, incubation time was increased to 2 hours.

Initially, G418 (Neomycin) was used as positive control. Neomycin is an antibiotic commonly used in molecular biology as a selection agent for transfected cells. It is known to be toxic to the mammalian cells (such as MDA-MB-231 and HEK-293). Due to the variability of the results achieved with this compound, it was replaced by 30 % w/v Carboplatin, in DMSO, which was experimentally proved to be more reproducible.

Another problematic variable which may have a negative effect on the reproducibility of the assay is the biological variability within the same plate and among different plates: in order to avoid it and to reduce its effect on the final results, the layout of the plate was changed as shown in Figure 59.

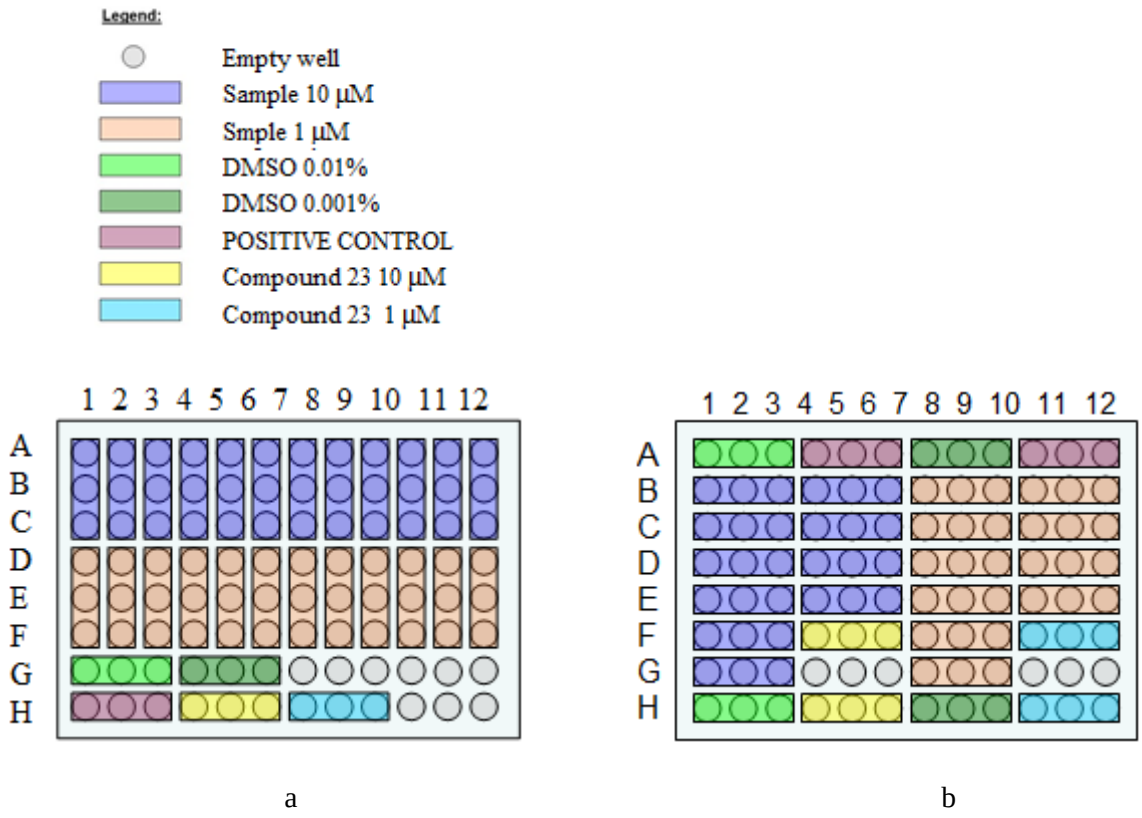


Figure 59. Layout of the 96-well plate in the cell titer blue assay. **a**) the original layout used; **b**) the optimised layout.

The main advantages on the new layout involve a different disposition of the seeding route which prevents to use a cell suspension with different concentrations between the initial and the final seeding. Using more technical repetitions for both controls and reference compound decreases the errors. Additionally, introducing variability in the organisation of the layouts helps to prevent variability on the results.

The results for compounds **23**, **186** and the new positive control (Carboplatin 30% w/v) are shown in in Figure 60, demonstrate that the optimisation of all the aforementioned parameters drastically improves reproducibility and decreases variability of the assay.

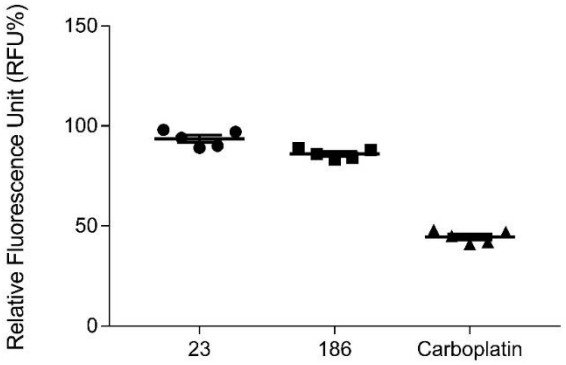


Figure 60. Results of the cell titer blue assay after optimisation of the general procedure for compounds **23**, **186** and Carboplatin as positive control. Cells were seeded at 100000 cells/well and cultured at 37 °C, 5% CO_2 for 24 hours. Cell titer blue reagent was incubated for two hours and absorbance was measured on the luminescence microplatereader, Clariostar (BMG Labtech). Individual measurements are normalised against negative control. Mean ± SEM, N=5, n=3.

This newly optimised protocol was then used to evaluate the cytotoxic profile of the novel compounds on both MDA-MB-231 and HEK-293 cell lines: these results are presented in section 5.1.

4.3 Enzyme-Linked Immunosorbent Assay

Disruption of Bcl-3/p50 binding is a new therapeutic option in the treatment of metastatic breast cancer, as discussed in section 1.7. One of the aims of this thesis was to develop an assay for the detection of the amount of formed Bcl-3:p50 complex to evaluate the activity of novel chemical entities for the treatment of breast cancer. Specifically, the aim was to detect, in the cell lysate, the amount of FLAG-tag-Bcl-3 protein and the amount of p50, which was bound to it.
This protein-protein interaction was investigated by Enzyme-Linked Immunosorbent Assay or ELISA.[8]

ELISA is a highly sensitive and precise plate-based assay, which involves the use of enzymes and antibodies to quantify the amount of an antigenic target substrate. The assay generally yields colourimetric or fluorimetric results which may then be interpreted either by eye or quantified by spectrophotometers.[9] It is a widely used assay to test protein-protein interactions (PPIs) and the ability of chemical compounds to inhibit these PPIs. Several types of ELISA exist, including direct ELISA, sandwich ELISA, competitive ELISA. Here, the optimisation process for the sandwich ELISA (direct and indirect) was investigated.[9]

To facilitate high throughput screening methods, the developed sandwich ELISA assay was supposed to be used in a 96-well format. In brief, the technical strategy to develop the ELISA assay was as

follows: the capture antibody (monoclonal, anti-FLAG antibody) is attached to the bottom of the well by passive absorption. These pre-coated plates are commercially available (Anti-FLAG, M2, pre-coated ELISA plates, Sigma Aldrich). For each cell line, both parental and wild type sub cell lines were tested at the same time: the parental cell line had an endogenous low level of Bcl-3 and it was used as negative control. The wild type cell line over-expressed FLAG-tag Bcl-3 protein. The lysate containing Bcl-3:p50 complex was prepared in non-denaturing conditions to preserve protein-protein interaction. After incubation of the lysate with the anti-FLAG capture antibody (to pull down FLAG-tag Bcl-3), a primary antibody is applied against either Bcl-3 or p-50. This primary antibody is then recognised by the addition of a secondary antibody which is conjugated to alkaline phosphatase. In the last step, the alkaline phosphatase then reacts with added para-nitrophenylphosphate, which leads to the development of a colorimetric change. After each step, washes are performed to remove unreacted material. The general reaction is reported in *scheme*[36].

I-Ab + Ag + AB + Anti-Ab*E + S → Read	*I-Ab* = Anti FLAG M2 mouse monoclonal antibody *Ag* = lysate *AB* = Antibody from another animal species as compared to I-Ab (rabbit polyclonal) *Anti-Ab*E* = anti-species antibody linked to enzyme *S* = para-nitrophenylphosphate.

Scheme[36]. Reaction scheme of sandwich Elisa.

Sandwich ELISAs may be distinguished in direct and indirect sandwich ELISAs, as represented in Figure 61. In both cases, the capture antibody is a monoclonal anti-FLAG antibody which pulls down only the non-endogenous Bcl-3 protein, tagged with the FLAG peptide (following its over-expression). In the direct ELISAs, a primary antibody against Bcl-3 is used to quantify the amount of FLAG-Bcl-3 attached on the capture antibody. Otherwise, in the indirect sandwich ELISA, a primary antibody against p50 is used: only p50 bound to FLAG- tag Bcl-3 is detected.

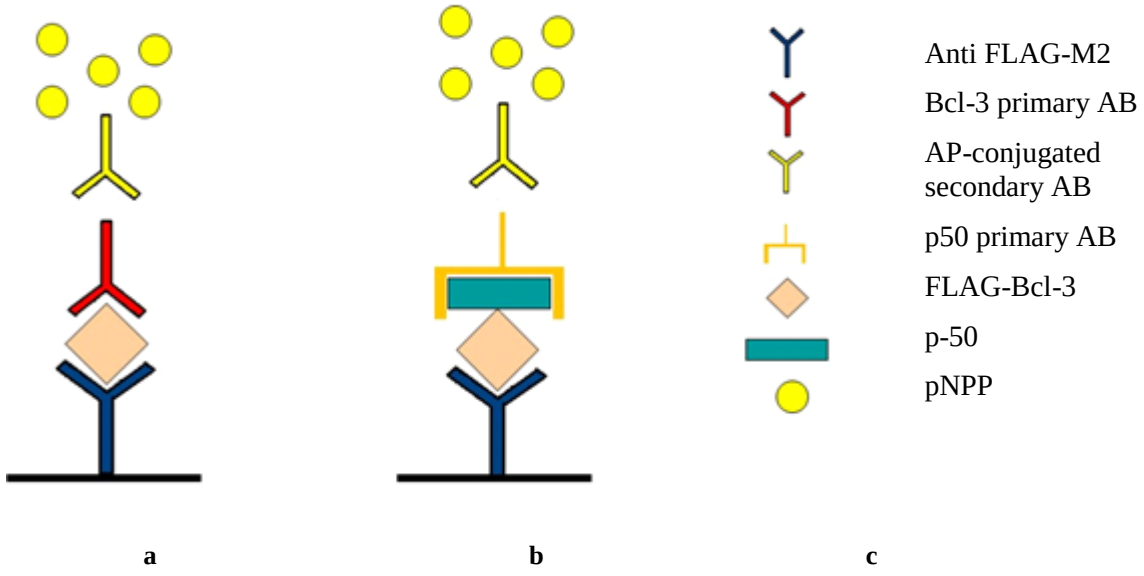


Figure 61. Schematic representation of sandwich ELISA assay. **a**) Direct sandwich ELISA. **b**) Indirect sandwich ELISA. **c)** legend: AB = antibody, AP = alkaline phosphatase, pNPP = para-nitrophenylphosphate.

Due to limited information available about the stoichiometry and composition of the Bcl-3:p50 or p52 complex and because of the limited information about the selective presence of p50 or p52, it was necessary also to detect p52, as it will be reported in experiments below.

Preliminary experiments detected both Bcl-3 and p50 in parental and wild type sub cell lines.[10] The expected signal was characterised by a gap in the absorbance between parental and wild type cell lines for both Bcl-3 and p50: parental cell line should give a low Bcl-3 and p50 absorbance, due to the absence of FLAG-tag Bcl-3 and hence due to the absence of Bcl-3:p50 complex, also p50 signal was expected to be low as the background one. In the wild type over-expressing cell line, the increased amount of FLAG-Bcl-3 should give a higher signal than in the parental cell line, and consequently an increased signal also for p50 (for further details on cell lines see section 7.1). This was due to Bcl-3 over-expression in the wild type cells (recombinant Bcl-3 was tagged with a FLAG peptide), which in turn increased nuclear levels of p50 and thus of the complex.

In the following sections, results from different parameters investigated in the course of the optimisation process will be presented for both Bcl-3 and p50.

Briefly, different cell lines were tested. For each cell lines, different conditions were investigated, such as primary antibodies, incubation times, washing and blocking solutions and stimulation of cells in presence of different stimuli.

The purpose of these series of experimental steps was to establish: 1) optimal binding conditions of the recombinant Bcl-3-FLAG tagged protein to the immobilised anti FLAG antibody substrate on the

plate; 2) optimal binding and detection of p50 protein, in complex with the FLAG-Bcl-3 protein, immobilised on the plate in the ELISA assay.

4.3.1 Optimisation of ELISA assay on HEK-293 cell line

4.3.1.1 Verification of standard ELISA conditions

First, MDA-MB-231, HEK-293 and their sub-cell lines (details in section 7.1) were tested to verify the Bcl-3 expression level. MDA-MB-231 cell line was used as a control for low endogenous Bcl-3 expression level, MDA-MB-231-WT as a Bcl-3 over-expressing cell line and the MDA-MB-231-ANK mutant as a Bcl-3 over-expressing clone not able to bind p50. The constitutive expression of GAPDH as housekeeping gene was detected as a loading control for all the following Western Blots.

HEK-293 (sample 1) and HEK-WT-7 (sample 4) cells were selected for optimisation of the ELISA assay because of their low endogenous Bcl-3 expression or Bcl-3 over-expression respectively, as confirmed by Western Blot shown in Figure 62.

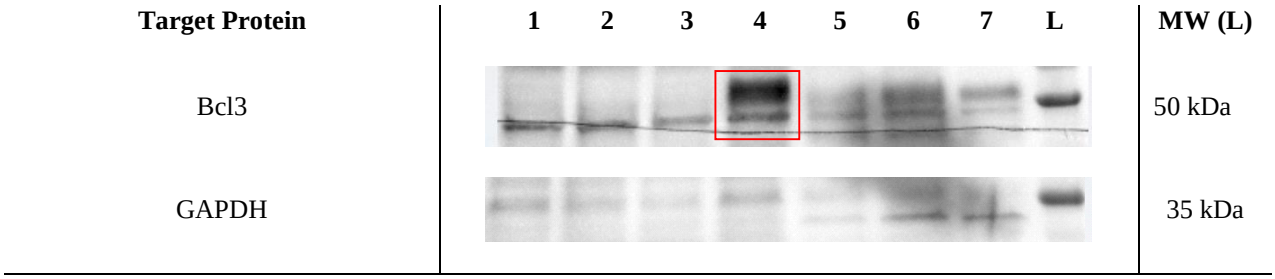


Figure 62. Western Blots for samples 1 – 7. Protocol in section 7.10. 1 = HEK-293, 2 = HEK-ANK, 3 = HEK-WT-4, 4 = HEK-WT-7, 5 = MDA-MB-231, 6 = MDA-MB-231-WT, 7 = MDA-MB-231-ANK, L=ladder. Antibody used: rabbit, polyclonal, Bcl-3 antibody (sc-185, SantaCruz); mouse, monoclonal GAPDH antibody (sc-32233, SantaCruz). Passage 1.

A pilot experiment was run to reproduce the previously reported experiments for Bcl-3 (experiment 1, 2, 3) and for p-50 (experiment 3, 4, 5) on both selected cell sub-lines, as represented in Figure 63. As previously stated, over-expression of Bcl-3 increases nuclear levels of p50. Hence, the main aim was to detect a low Bcl-3 signal for HEK-293 and to increase the Bcl-3 absorbance signal in the HEK-WT-7 (over-expressing cell sub-line). Moreover, the second aim was to reduce the p-50 absorbance signal in the HEK-293 (not over-expressing Bcl-3 cell sub-line) and to improve the detected signal for p50 in HEK-WT-7. Original conditions were used. [10] For Bcl-3 detection, as reported in Figure 63.a, although the difference between the detected absorbance in the two cell lines (parental and wild

type) reflects the expected signal (low absorbance in the parental cell line, high absorbance in the wild type), it is not significant. For p50 detection, as reported in Figure 63.b, the detected absorbance is not reproducible and does not correlate to the expected signal: in the parental cell line a low absorbance is predicted, due to the absence of FLAG-Bcl-3, hence there is no binding with p50; additionally, in the wild type cell line, due to the increased amount of FLAG-Bcl-3, the detection of p50 should give a higher signal. Conversely, the p50 detected signal is high in both cell lines and the difference between the two cell lines is not sharp.

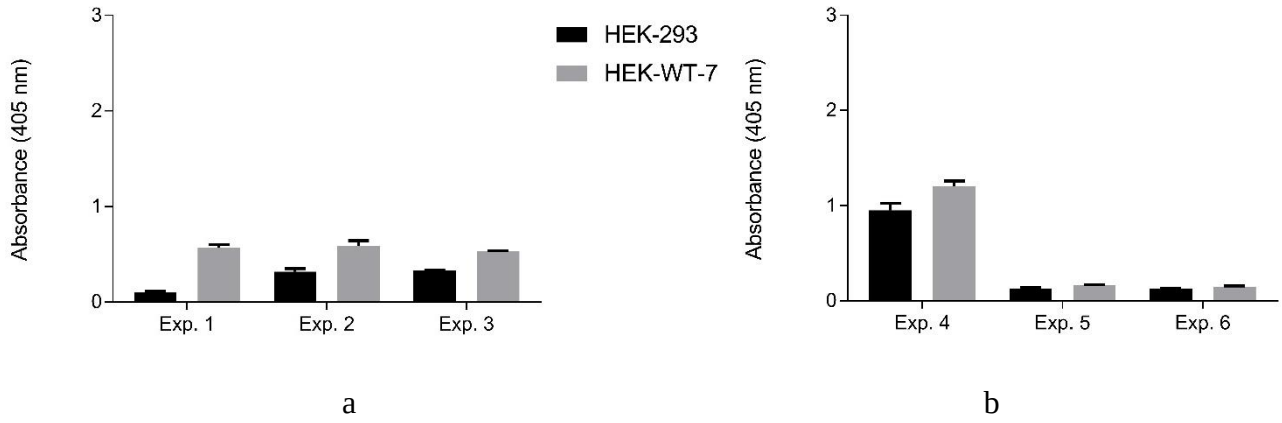


Figure 63. Sandwich ELISA assay results. a) Direct sandwich ELISA detection of Bcl-3 in HEK-293 and HEK-WT-7 cells, cultured in 300 µg/mL of G-418. Experiment (Exp.) 1, 2 and 3 represent three independent repetitions of the same original conditions (protocol in section 7.11, primary antibody: rabbit polyclonal, sc-185, Santa Cruz). b) Indirect sandwich ELISA detection of p50 in HEK-293 and HEK-WT-7 cells, cultured in 300 µg/mL of G-418. Experiment (Exp.) 4, 5 and 6 represent three independent repetitions of the same original conditions (protocol in section 7.11, primary antibody: rabbit, polyclonal, ab7549, Abcam).[10] Mean + SEM, N=1, n=3.

Hence, it was necessary to investigate different parameters to optimise the signal for both proteins.

4.3.1.2 Detection of Bcl-3: direct sandwich ELISA

In the optimisation of the ELISA conditions to detect Bcl-3, the first considered parameter is lysate concentration. Even though established previously,[10] different concentrations (1 µg/mL, 10 µg/mL, 100 µg/mL, 200 µg/mL) were tested for completion. Results are reported in Figure 64. At lower concentrations an increase of absorbance may occur due to cross reactivity of the primary antibody with the capture antibody on the plate, due to a lack in the saturation of putative paratope on Bcl-3 protein. Between 10 and 100 µg/mL, a plateau in the absorbance is observed and this corresponds to the maximum amount of lysate may be seeded in each well. At higher concentrations, such as 100 or

200 µg/mL, there is a slight increase in absorbance measurement, but due to a lack of a steady logarithmic increase in absorbance, a saturation point was reached at 10 µg/mL.

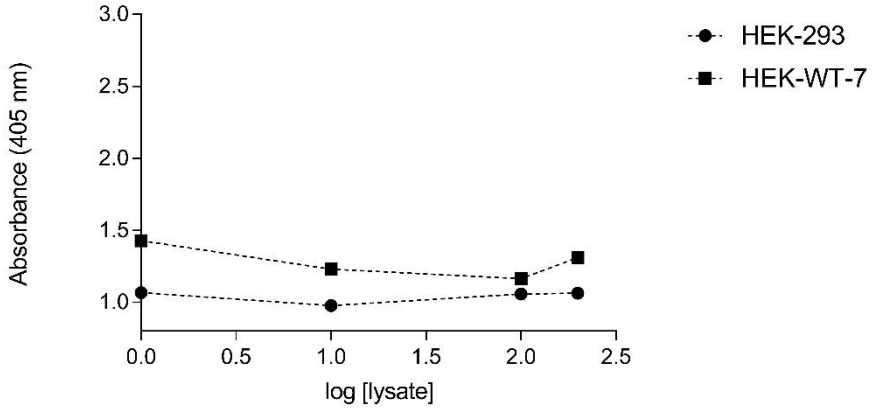


Figure 64. Direct sandwich ELISA to detect Bcl-3 over a range of protein concentration. HEK-293 and HEK-WT-7 cells, cultured in 300 µg/mL of G-418 were seeded on the ANTI-FLAG pre-coated plate at different concentration (1 µg/mL, 10 µg/mL, 100 µg/mL, 200 µg/mL) (protocol in section 7.11, primary antibody: rabbit polyclonal, sc-185, Santa Cruz). Mean + SEM, N=1, n=3.

Furthermore, a series of different conditions were investigated, as summarised in Table 8.

Table 8. Summary of different conditions in experiment 7 - 11.

Exp.	Primary antibody information	Lysate dilution buffer	Antibody dilution buffer	Stopping solution	Washes
7	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin (Lot#K2713)	TBS/T 0.5%	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%
8	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	TBS/T 0.5%	TBS/T 0.5% +Triton X100 0.3%	NaOH 3M	TBS/T 0.5%
9	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	TBS/T 0.5%	TBS/T 0.5%	Na_2CO_3 2M	TBS/T 0.5%
10	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	TBS/T 0.5%	PBS 1X	NaOH 3M	TBS/T 0.5%
11	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	TBS/T 0.5%	TBS/T 0.5%	NaOH 3M	PBS/T

Results are reported in Figure 65. The control did not contain lysate to check for capture and primary antibody cross reactivity (this was valid for any control used in the following sections as well). In experiment 7, the antibody was the same as the one used in experiment 1, 2 and 3, but from a different lot ((C-14), sc-185, Lot#K2713). In previous experiments, the antibody was diluted in TBS/T 0.5%, while in experiment 8 it was diluted in TBS/T 0.5% Triton-X-100 0.3% : this non-ionic surfactant is commonly used in immunohistochemistry for blocking. However, the signal for HEK-293 cells is a slightly higher than in the other experiments and the difference between the parental and the wild type cell lines is still not large, suggesting that the use of TBS/T 0.5% Triton-X-100 0.3% does not improve the absorbance signal.

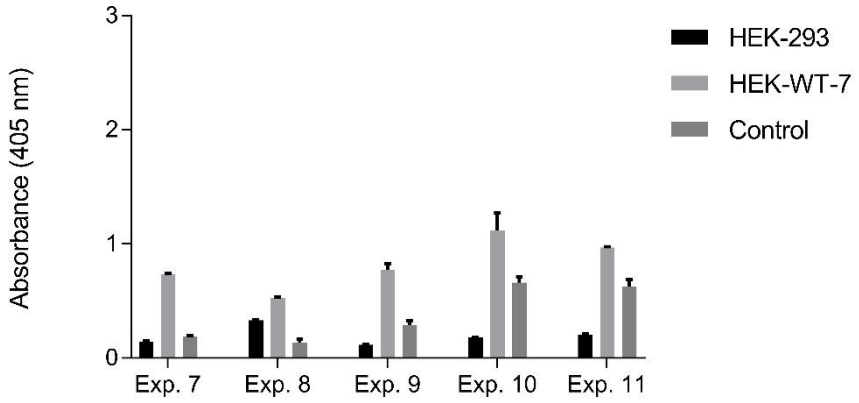


Figure 65. Direct sandwich ELISA to detect Bcl-3: different tested parameters, listed in Table 8. Cells used: HEK-293 and HEK-WT-7, cultured in 300 µg/mL of G-418 (protocol in section 7.11). Mean + SEM, N=1, n=3.

In experiment 9, a different stopping solution was used to stop the reaction between the secondary alkaline phosphatase-conjugated antibody and para-nitrophenylphosphate solution: aqueous NaOH solution (3M) used in experiments 1 - 3 was replaced with Na_2CO_3 (2M). However, there was no observable difference in the achieved signal in response to the different stopping solutions; indeed, the background signal of the control was even visible higher than before.

Experiment 10 investigated the effect of PBS (1X) when used to dilute the primary antibody: while in experiments 1 -3, TBS/T 0.3% was used to dilute the antibody, here the use of PBS (1X) increases the Bcl-3 signal, but also the one detected for the control.

Experiment 11 evaluated the effects of washes solution on absorbance reading. Instead of TBS/T 0.5% solution, phosphate buffered saline (PBS) solution was used to perform the washes, after each

incubation step of the general protocol to remove the unreacted material. As shown in Figure 65, the Bcl-3 signal is 1.5 times higher when washing with PBS instead of TBS/T 0-3%. The main difference between PBS and TBS/T 0.3% is the ionic strength.[11] The ionic strength may influence the rate of the reaction between antigen and antibody. Buffers with low ionic strength may contribute to non-specific bindings of the conjugate to the capture antibody, while increase concentration of ionic strength may be used to minimise non-specific interactions, but further increases in ionic strength may cause decrease in binding affinity between antibodies and coated antigens, up to hindrance of antibody-antigen binding completely. In addition, when specific binding between antigen/antibody is achieved in a solution with low ionic strength, subsequent washes with a low-ionic-strength buffer solution leads to complete dissociation of the complexes (weak binding). On the other hand, if the specific binding reaction is performed in a high ionic-strength solution, most of the binding persists after the washing with low ionic strength solution. At pH 7.4, the ionic strength of PBS (0.1 M, pH 7.4) is 0.27 M, while the ionic strength of TBS/T (0.1 M, pH 7.4) is 0.084 M. In general, in experiment 10 and 11 while the Bcl-3 signal increases, the signal for control background increases as well, suggesting that the increase in Bcl-3 detection may be only an artefact of increased background colour. Anyway, the absorbance detected in experiment 10 was the highest achieved for Bcl-3 detection. Thus, further investigations determined whether it was possible to reduce the increased non-specific binding for control. To check whether the dilution buffer may interfere with the protein-protein interaction in the ELISA assay, a few more conditions in deep analysis were checked. Dilution of the cell lysate with PBS and the use of a blocking agent were investigated in two different incubation times: 60 and 90 minutes. Conditions for experiments 12-14 are listed in Table 9.

Table 9. Summary of different conditions in experiment 1, 12 - 14.

Exp.	Primary antibody information	Lysate dilution buffer	Antibody dilution buffer	Stopping solution	Washes
1	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin (Lot#K2713)	TBS/T 0.5%	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%
12	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	PBS 1X	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%
13	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	PBS 1X+ 1% BSA	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%
14	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	PBS 1X+ 5% BSA	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%

In Figure 66, experiment 12 shows the effect of using PBS to dilute the lysate, while experiment 13 and 14 evaluated the use of 1% and 5% of bovine serum albumin (BSA) in PBS, respectively.

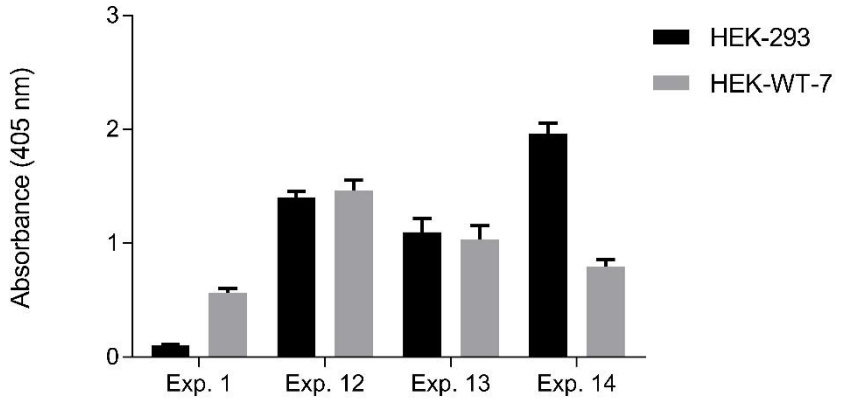


Figure 66. Direct sandwich ELISA to detect Bcl-3: different tested parameters, listed in Table 9. HEK-293 and HEK-WT-7 were cultured in 300 µg/mL of G-418. Cell lysate was seeded at 10 µg/mL on the ANTI-FLAG M2 pre-coated plate and incubated at 37 °C for one hour (according to the general protocol in section 7.11). Mean + SEM, N=1, n=3.

The BSA is a widely used blocking agent which helps to prevent non-specific binding in several immunoassays. The dilution of lysate in PBS (experiment 12) increased the detected absorbance signal, but due to cross-reactivity, there was no difference in the signal between HEK-293 and HEK-WT-7. Moreover, the use of 1% BSA (experiment 13) did not improve the difference in the detected signals for Bcl-3 in the two cell lines. On the other hand, higher concentration of BSA (5%, experiment 14) badly influences the results: as shown in Figure 66, the absorbance signal is higher for the parental cell line (low endogenous Bcl-3 expression level) than in the over-expressing cell line, suggesting that the blocking solution increases the non-specific binding.[12] Although BSA is widely used, different blocking solutions (casein or skimmed milk powder) need to be investigated to check whether they may improve the detection for Bcl-3. Dilution of cell lysate in PBS and incubation with 1% BSA were performed increasing incubation time from 60 minutes to 90 minutes incubation of lysate on the plate (conditions listed in Table 10). The main reason was to address the question whether the lack of a different absorbance between the two different sub lines is due to a short incubation time, results are presented in Figure 67.

Table 10. Summary of different conditions in experiment 1, 15, 16. In Experiment 1 (Exp.1) cell lysate was seeded at 10 µg/mL on the ANTI-FLAG M2 pre-coated plate and incubated at 37 °C for one hour (according to the general protocol in section 7.11). In Experiments (Exp.) 15 and 16, cell lysate was seeded at 10 µg/mL on the ANTI-FLAG M2 pre-coated plate and incubated at 37 °C for 90 minutes (according to the general protocol in section 7.11).

Exp.	Primary antibody information	Lysate dilution buffer	Antibody dilution buffer	Stopping solution	Washes
1	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin (Lot#K2713)	TBS/T 0.5%	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%
15	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	TBS/T 0.5%	PBS 1X	NaOH 3M	TBS/T 0.5%
16	rabbit polyclonal, sc-185, SantaCruz, epitope corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	PBS 1X + 1% BSA	TBS/T 0.5%	NaOH 3M	TBS/T 0.5%

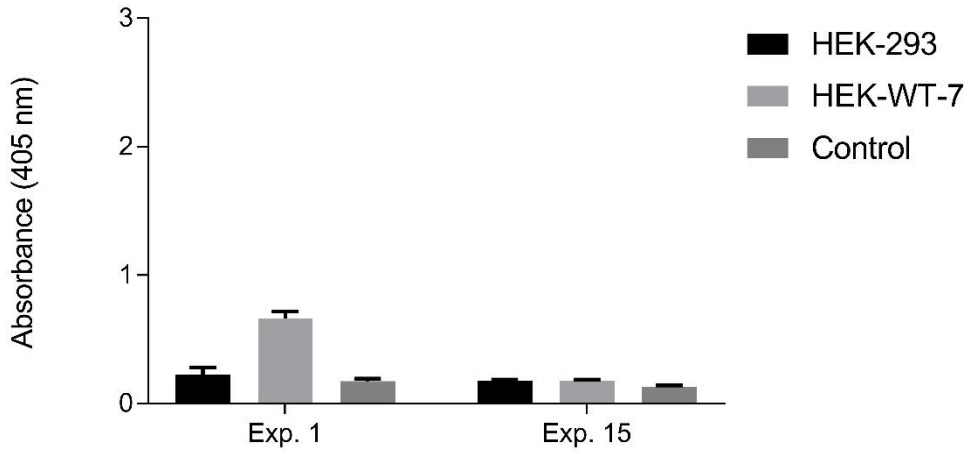


Figure 67. Direct sandwich ELISA to detect Bcl-3: different tested parameters, listed in Table 10. HEK-293 and HEK-WT-7 were cultured in 300 µg/mL of G-418. Cell lysate was seeded at 10 µg/mL on the ANTI-FLAG M2 pre-coated plate and incubated at 37 °C for 90 minutes (according to the general protocol in section 7.11). Mean +SEM, N=1, n=3.

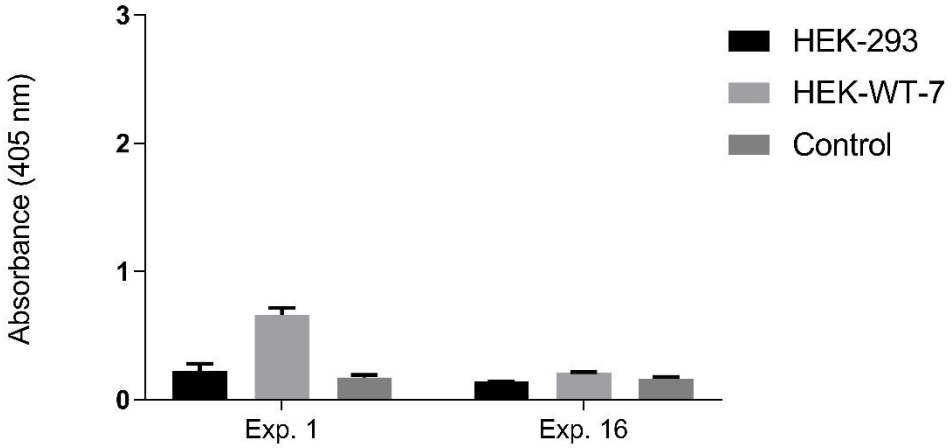


Figure 68. Direct sandwich ELISA to detect Bcl-3: different tested parameters, listed in Table 10. HEK-293 and HEK-WT-7 were cultured in 300 µg/mL of G-418. Cell lysate was seeded at 10 µg/mL on the ANTI-FLAG M2 pre-coated plate and incubated at 37 °C for 90 minutes (according to the general protocol in section 7.11). Mean + SEM, N=1, n=3.

The 5% BSA was not investigated due to previous unfavourable results in Figure 66. The dilution with PBS and a 90-minutes incubation of lysate on the plate (experiment 15, Figure 67) results in a levelling off the absorbance detected for both Bcl-3 and p50 in the two cell lines. The use of 1% BSA (experiment 16, Figure 68) does not improve neither the absorbance for both proteins nor the difference in the signal between the parental and the wild type cells.

Due to the non-satisfying results for Bcl-3, dilution of cell lysate in BPS or the use of BSA as blocking solution may not be used in this ELISA protocol.

To increase the signal for Bcl-3, cell stimulation by TNF-α activation to up-regulate the NF-κB pathway and thus the Bcl-3:p50 complex formation, was investigated.[13] Recombinant human- TNF-α at a concentration of 10 ng/mL was added to the cells before lysate preparation. Incubation times with TNF-α was not determined in literature for the specific cell lines used. Because of the established incubation time in HEK-293 cell line for lipopolysaccharide (LPS), which stimulates the same NF-κB pathway cascade, the published incubation times for this ligand was used as a reference point.

Thus, two different incubation times were investigated, as reported in Figure 69: 3 hours (experiment 17) and 18 hours (experiment 18) incubation time, to assess both short and long-term incubation.

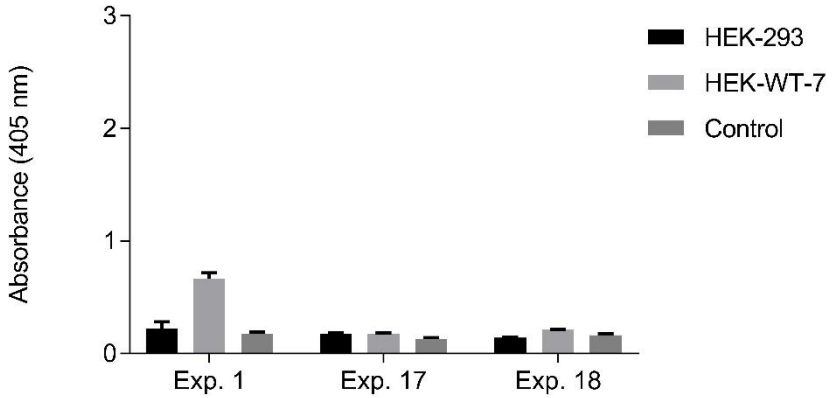


Figure 69. Direct sandwich ELISA to detect Bcl-3 after TNF-α stimulation. Cells used: HEK-293 and HEK-WT-7, cultured in 300 µg/mL of G-418. Cells were stimulated for 3 hours (experiment 17) and 18 hours (experiment 18) with 10 ng/mL TNF-α stimulation, prior to lysate preparation Primary antibody used: rabbit, polyclonal Bcl-3 antibody (sc-185, SantaCruz). Mean + SEM, N=1, n=3.

However, while there was no effect on the signal in HEK-293 cells or that background control, stimulation with TNF-α of Bcl-3 over-expressing HEK-WT-7 cells actually leads to a drastically decrease of signal for Bcl-3, in both experiments.

Finally, two different Bcl-3 antibodies were tested. They are listed in Table 11 and results are shown in Figure 70.

Table 11. Summary of different antibodies in experiment 1, 19, 20.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
1	SantaCruz	sc-185	rabbit polyclonal	corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
19	SantaCruz	sc-130380	rabbit polyclonal	corresponding to amino acids 301-446 mapping at the C-terminus of Bcl-3 of human origin	1:200
20	Proteintech	AP-23959-1AP	rabbit polyclonal	Bcl-3 fusion protein	1:200

Experiment 19 improves the results for Bcl-3 detection, compared to experiment 1. Hence, signal intensities were greatly enhanced when the experiment was performed using this latter antibody.

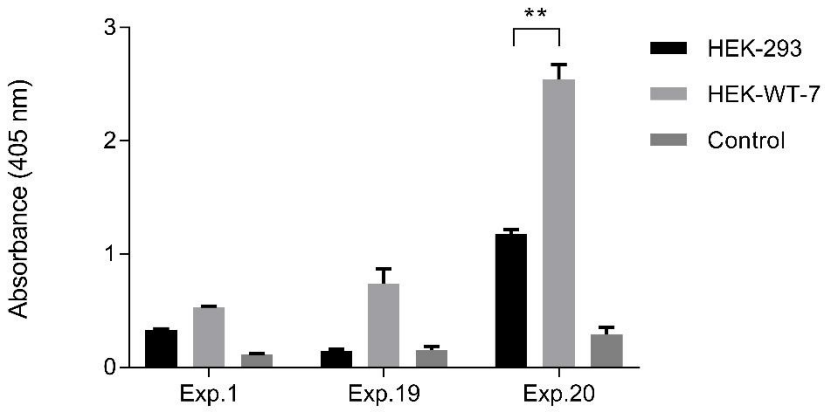


Figure 70. Direct sandwich ELISA to detect Bcl-3 using different Bcl-3 directed antibodies. Cells used: HEK-293 and HEK-WT-7, cultured in 300 µg/mL of G-418. Antibody used listed in Table 11. Mean + SEM, N=1, n=3. $p < 0.001$ (Two-Way Anova, Tukey's multiple comparisons test).

The last parameter to investigate was the actual incubation of the cells with the evaluated compounds to determine if the presence of compounds affected Bcl-3 detection. Previously, compounds were incubated for 24 hours with cells prior to cell lysate preparation.[10] Here, one hour incubation of compound **23** at 10 µM with cells prior to preparation of lysate were investigated. Resulted are displayed in Figure 71. A 1 hour incubation of compound **23** with cells did not affect badly the detection signal for Bcl-3, when compared to the absorbance obtained from cells which were cultured for 24 hours in presence of the test compound at 10 µM. The two procedures are equivalent, but the sharp decrease of the incubation time is greatly beneficial in the development of a high-throughput screening assay, where several compounds must be tested.

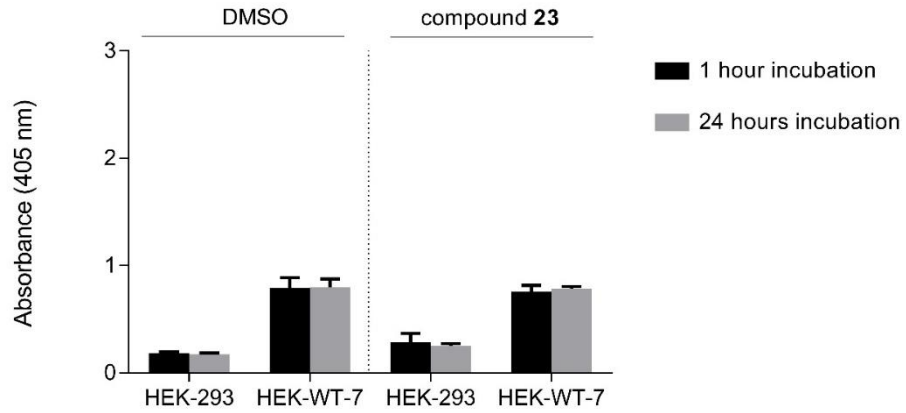


Figure 71. Direct sandwich ELISA to detect Bcl-3 in different incubation times of compounds with cells, prior to cell lysate preparation. Cells used: HEK-293 and HEK-WT-7, cultured in 300 µg/mL of G-418. Cells were cultured in presence of compound **23** (10 µM) for one hour or 24 hours prior of lysate preparation. Antibody used: rabbit, polyclonal Bcl-3 antibody (23959-1-AP, Proteintech). Mean + SEM, N=1, n=3.

Following all these optimisation steps, the best possible conditions were chosen as the following: TBS/T 0.5% dilution of protein lysate and antibody, TBS/T 0.5% washes, aqueous NaOH (2M) as stopping solution, and the use of the new antibody 23959-1-AP as primary antibody for Bcl-3.

4.3.1.3 Detection of p50: indirect sandwich ELISA

Once conditions to detect Bcl-3 were optimised, a similar process was applied to this assay to enhance the detection of p50. As reported in Figure 72, the signal for p50 across the three independent repetitions of the same experimental conditions was not reproducible. Additionally, whereas in experiment (Exp.) 4 the absorbance is approximately 1, in Exp. 5 and 6, it is not different from the absorbance detected usually for the background control (experiment 4-6).

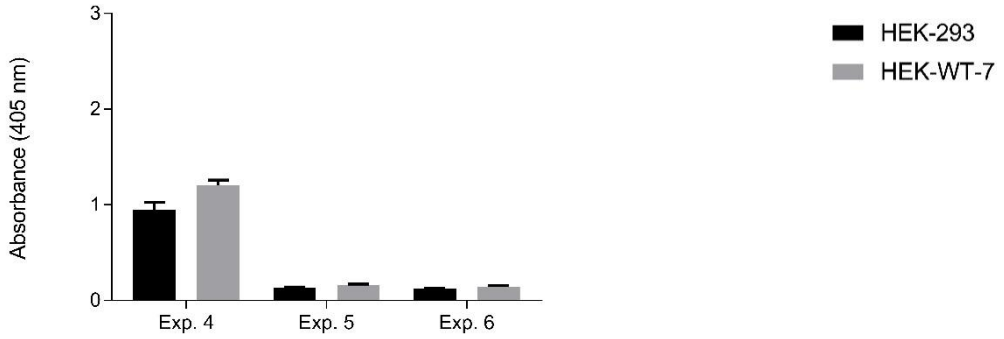


Figure 72. Indirect sandwich ELISA detection of p50 in HEK-293 and HEK-WT-7 cell lines. Cells were cultured in 300 µg/mL of G-418. Experiment (Exp.) 4, 5 and 6 represent three independent repetitions of the same original conditions (protocol in section 7.11, primary antibody: rabbit, polyclonal, ab7549, Abcam).[10] Mean + SEM, N=3, n=3.

Parameters such as dilution buffers for both lysate and antibody or different wash solutions were not considered because they did not affect results for Bcl-3 detection. The parameters tested to check whether they affect p50 detection were: different antibodies mapping different regions of the target protein and stimulation of Bcl-3:p50 complex formation by TNF-α activation.

Different antibodies were tried, as summarised in Table 12. Working dilutions were selected according to manufacturer's instructions. Results are reported in Figure 73.

Table 12. Different antibodies in the indirect sandwich ELISA for p50 detection, experiments (Exp.) 21 - 25.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
21	SantaCruz	sc-101744	rabbit polyclonal	phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
22	SantaCruz	sc-1190	goat polyclonal	C-terminus of NF-κB p50 of human origin	1:200
23	SantaCruz	sc-114	rabbit polyclonal	NLS region of NF-κB p50 of human origin	1:200
24	SantaCruz	sc-114x	rabbit polyclonal	NLS region of NF-κB p50 of human origin	1:200
25	Proteintech	14220-1AP	rabbit polyclonal	NF-κB1 GST fusion protein	1:100

However, independently of the used antibody, the p50 signal was still inconclusive, as the obtained results did not correspond to the original experiment 1 and did not show any differences between HEK-293 and Bcl-3 overexpressing HEK-WT-7, as reported in Figure 73 This may be due to either the disruption of protein-protein interaction due to the several, albeit necessary, washing steps or due to a low concentration of p50 in unstimulated cells prior to lysate preparation.

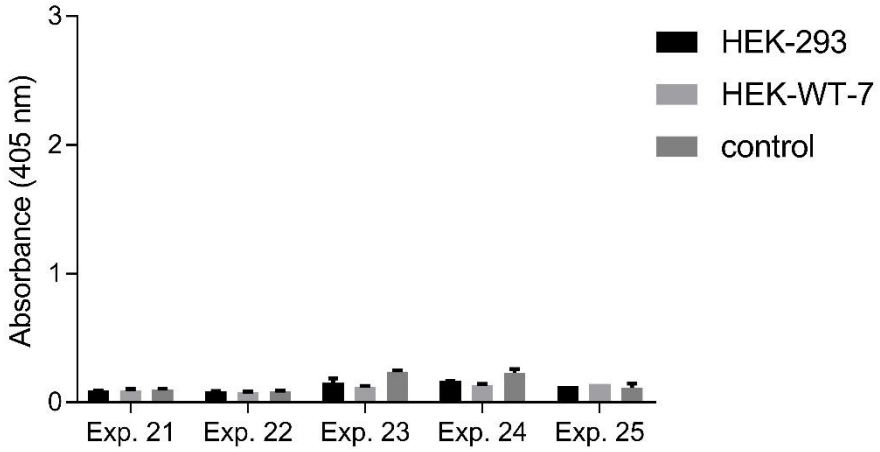


Figure 73. Indirect sandwich ELISA to detect p50 in HEK-293 and HEK-WT-7 cell lines. Cells were cultured in 300 µg/mL of G-418. Experiment (Exp.) 21 – 25 tested different p50 primary antibody (protocol in section 7.11, listed in Table 12).[10] Mean + SEM, N=3, n=3.

To investigate the possibility of reduced p50 expression and overcome this potential shortcoming, Bcl-3:p50 complex formation was stimulated with TNF-α treatment of cells.[13] Cell lysates were prepared after 3 and 18 hours of incubation with TNF-α (10 ng/mL), as detailed above for Bcl-3.

Different antibodies were used to probe for p50 expression, as listed in Table 13. Experiments for the 3h TNF-α incubation time point are reported in Figure 74.

Table 13. Indirect sandwich ELISA: different antibodies to detect p50 after 3-hours TNF-α (10 ng/mL) stimulation of cells. HEK-293 and HEK-WT-7 cells, were cultured in 300 µg/mL of G-418.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
26	SantaCruz	sc-101744	rabbit polyclonal	phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
27	SantaCruz	sc-1190	goat polyclonal	C-terminus of NF-κB p50 of human origin	1:200
28	SantaCruz	sc-114	rabbit polyclonal	NLS region of NF-κB p50 of human origin	1:200
29	Abcam	ab31412	rabbit polyclonal	endogenous levels of NF-κB p105/p50 protein around Serine 907	1:200
30	Abcam	ab28849	rabbit polyclonal	phosphopeptide derived from human NF-κB p105/p50 around the phosphorylation site of Serine 337	1:200

Results for these attempts are displayed in Figure 74. No improvement of the signal for p50 was observed in any of the performed experiments.

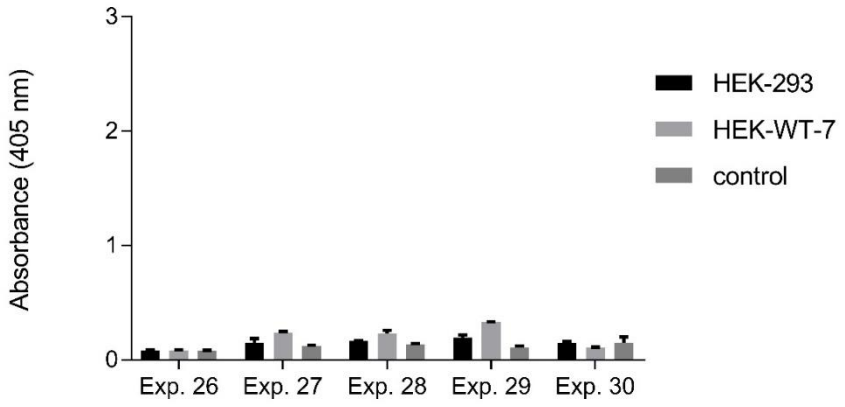


Figure 74. Indirect sandwich ELISA to detect p50 after 3 hours TNF-α stimulation. HEK-293 and HEK-WT-7 cells were cultured in 300 µg/mL of G-418 and stimulated for 3 hours with TNF-α (10 ng/mL) prior to lysate preparation (ELISA usual protocol, reported in section 7.11). Mean + SEM, N=1, n=3.

Lysate prepared from cells that were treated with TNF-α (10 ng/mL) for 18 hours were probed for p50 using the antibodies detailed in Table 14.

Table 14. Indirect sandwich ELISA: different antibodies to detect p50 after 18-hours TNF-α (10 ng/mL) stimulation of cells. HEK-293 and HEK-WT-7 cells, were cultured in 300 µg/mL of G-418.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
31	SantaCruz	sc-101744	rabbit polyclonal	corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
32	SantaCruz	sc-1190	goat polyclonal	mapping at the C-terminus of NF-κB p50 of human origin	1:200
33	SantaCruz	sc-114	rabbit polyclonal	mapping within the NLS region of NF-κB p50 of human origin	1:200

Again, in these experiments (Figure 75), a signal for p50 different from the background control could not be achieved.

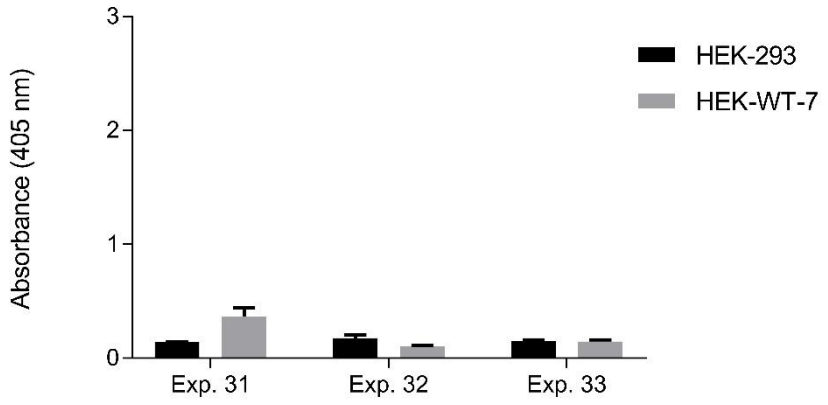


Figure 75. Indirect sandwich ELISA to detect p50 after 18 hours TNF-α stimulation. HEK-293 and HEK-WT-7 cells were cultured in 300 µg/mL of G-418 and stimulated for 3 hours with TNF-α (10 ng/mL) prior to lysate preparation (ELISA usual protocol, reported in section 7.11). Mean + SEM, N=1, n=3.

After checking different conditions and different antibodies, it was not possible to detect p50 protein. Possible reasons may be the disrupt of the Bcl-3/p50 binding because of any of the steps involved in the ELISA protocol or the Bcl-3 expression level in the cells: depending on the amount of recombinant FLAG-tagged Bcl-3 protein, if it is not highly over-expressed, it is possible that the amount of formed complex is too low to be detectable with the ELISA assay.

4.3.1.4 Detection of p52: indirect sandwich ELISA

Due to the limited information available in the literature whether the complex formation may involve p50 or p52, it was worth try to detect p52 instead of p50. The general method is essentially the same as the one described in section 4.3 for the p50 detection.

Three different antibodies were used to detect p52, as listed in Table 15.

Table 15. Indirect sandwich ELISA: summary of antibody against p52 in HEK-293 and HEK-WT-7 cell lines. HEK-293 and HEK-WT-7 cells, cultured in 300 µg/mL of G-418.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
33	SantaCruz	sc-848	rabbit polyclonal	amino acids 1-447 representing full length NF-κB p52 of human origin	1:200
34	SantaCruz	sc-7386	mouse monoclonal	amino acids 1-447 representing full length NF-κB p52 of human origin	1:200
35	CellSignalling	4882s	rabbit polyclonal	detects endogenous levels of p100, the precursor, and p52, the mature form of NF-κB2	1:100

Results are presented in Figure 76. The signal detected for p52 was of the same magnitude of the ones obtained for p50, suggesting that also for p52 an optimisation process of the general protocol of the indirect sandwich ELISA is necessary.

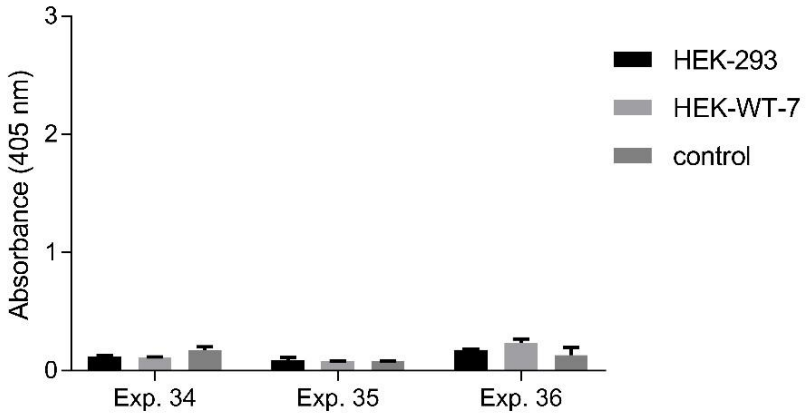


Figure 76. Indirect sandwich ELISA to detect p52 with different antibodies. HEK-293 and HEK-WT-7 cells, cultured in 300 µg/mL of G-418 (protocol in section 7.11). Mean + SEM, N=1, n=3.

4.3.1.5 Conclusions

Cells used in experiment reported in Figure 71 shown a decreased absorbance signal for the detection of Bcl-3protein. Western Blot experiments were performed to ascertain the presence of Bcl-3, p50 and p52 in tested cells.

Western Blot, reported in Figure 77 , shows a dramatically decrease in Bcl-3 over-expression level.

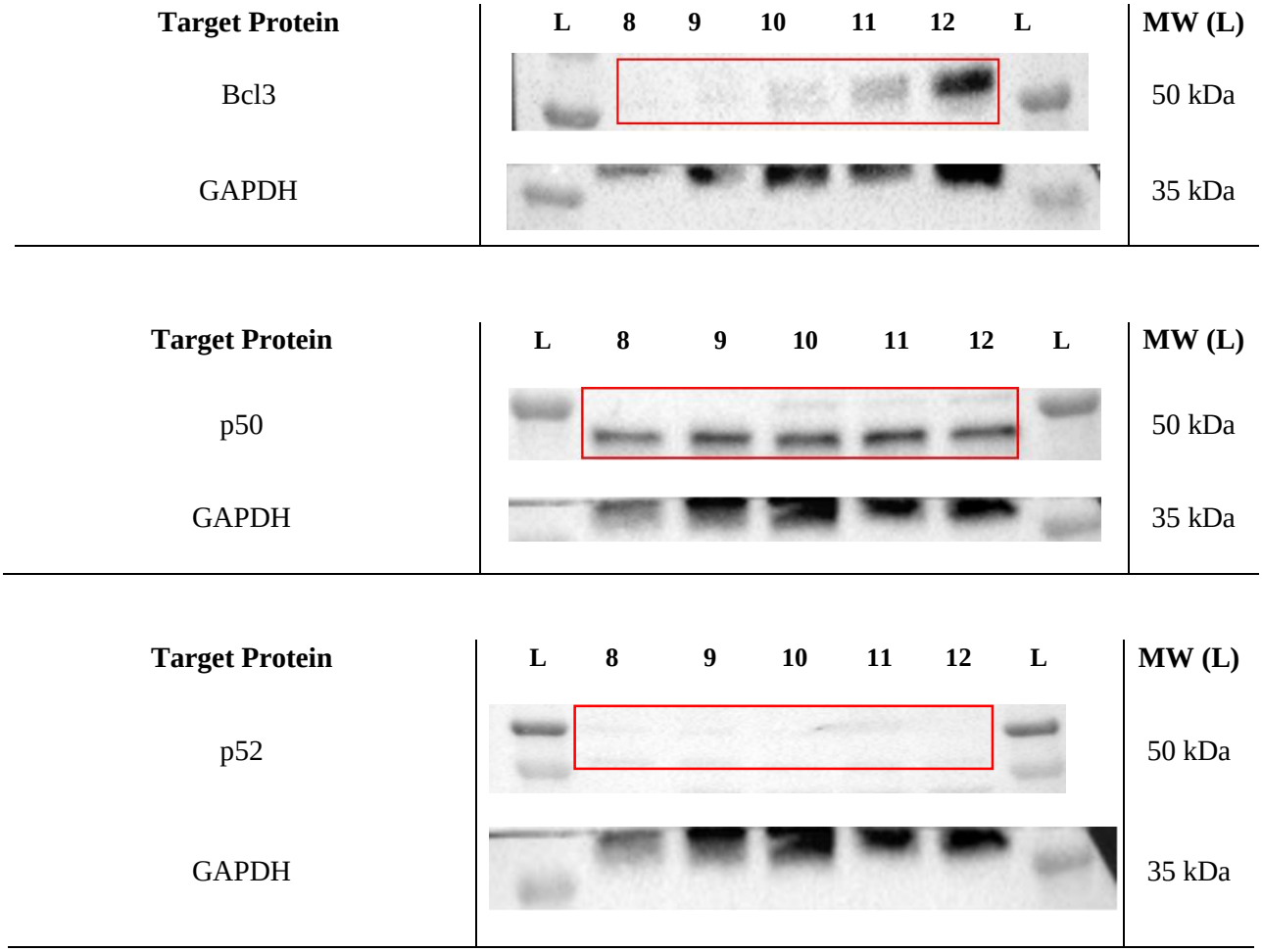


Figure 77. Western Blots for samples 8 – 12. 8 = HEK-293, 9 = HEK-WT-7, 10 = MDA-MB-231, 11 = MDA-MB-231-WT, 12 = MDA-MB-231-ANK, L = ladder. Protocol in section 7.10. Antibody used: rabbit polyclonal, Bcl-3 antibody (23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000). Passage 5.

In denaturing conditions, such as the ones used in the employed Western Blot protocol (see section 7.10), p50 was detectable in the investigated cell-lines. Conversely, no p52 was detected.

Taken together the results from the ELISA and Western Blot experiments suggest that either p50/p52 does not bind to the FLAG-tag-Bcl-3 in an amount which can be detected by ELISA or that during the ELISA, the Bcl-3/p-50 binding is disrupted by the experimental conditions (washing steps,

buffers, incubation times). Due to the loss of Bcl-3 expression after few cell passages (cells were checked by Western Blots every 5 passages), as established above, another cell lines was investigated, SW-480 (details in section 7.1, 7.2). These experiments will be further discussed in the next sections.

4.3.2 SW-480 cell line

4.3.2.1 Detection of Bcl-3 and p50

Human colon adenocarcinoma SW-480 cells were successfully transfected to over-express Bcl-3 in the sub-line SW-480-WT. They were a kind gift from Professor Ann Williams, Bristol University. At first, the suitable concentration of selective antibiotic (G-418) was not known, hence the cell lines were cultured at two different concentrations: 500 µg/mL and 1000 µg/mL of selection agent. At 500 µg/mL of G-418, SW-480 cells and the Bcl-3 over-expressing sub-line expresses lower levels of Bcl-3 than at 1000 µg/mL of G-418, always comparing against a control cell line (HT29, colorectal adenocarcinoma, characterised by endogenous high Bcl-3 level).[14] Expression level of p50 is comparable in all the cell lines, as shown in Figure 78.

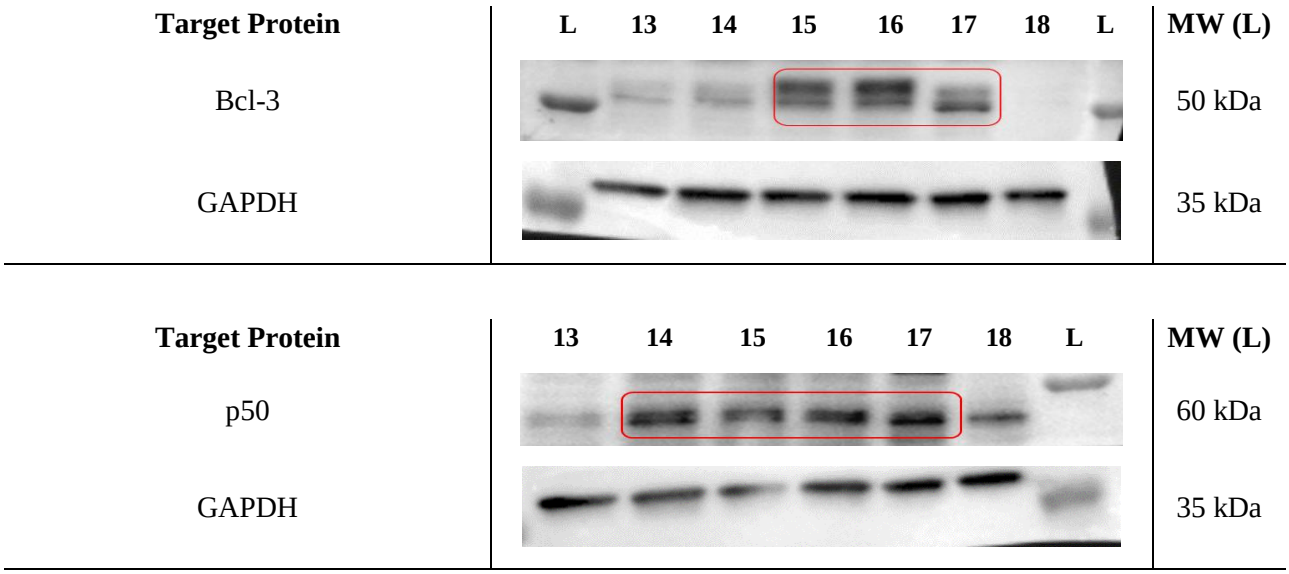


Figure 78. Western Blots for samples 13 – 18. L=ladder, 13=SW-480 (500 µg/mL of G-418), 14= SW-480-WT (500 µg/mL of G-418), 15= SW-480 (1000 µg/mL of G-418), 16=SW-480-WT 1000 µg/mL of G-418), 17=HT29, 18=MDA-MB-231. Protocol in section 7.10. Antibody used: rabbit polyclonal, Bcl-3 antibody (23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000).

After the initial Western Blot characterisation, these cell lines were used for testing the ELISA protocol. The "standard" Bcl-3 antibody (23959-1-AP) and three different p50 antibodies were tested, as schematised in Table 16.

Table 16. Summary of different antibodies used for direct and indirect sandwich ELISA to detect Bcl-3 and p-50 in SW-480 and SW-480-WT cells. Experiments (Exp.) 37-40 were performed on lysate from cells cultured in the presence of 500 µg/mL G-418 and Exp. 41-44 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418.

Exp.	**Supplier**	**Antibody catalogue number**	**Species**	**Epitope**	**Working dilution**
37/41	Proteintech	23959-1-AP	rabbit polyclonal	Bcl-3 GST fusion protein	1:200
38/42	Abcam	ab-7549	rabbit polyclonal	phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
39/43	Abcam	ab-54162	rabbit polyclonal	Recombinant fragment corresponding to Human NF-κB p105/ p50 (internal region of the p50 subunit)	1:200
40/44	Abcam	ab-14220	rabbit polyclonal	NF-κB1 GST fusion protein	1:200

Cells cultured in 1000 µg/mL of selective antibiotic have a slightly better detection profile for Bcl-3 and p50 antibody (Exp. 40 and 41), as reported in Figure 79.

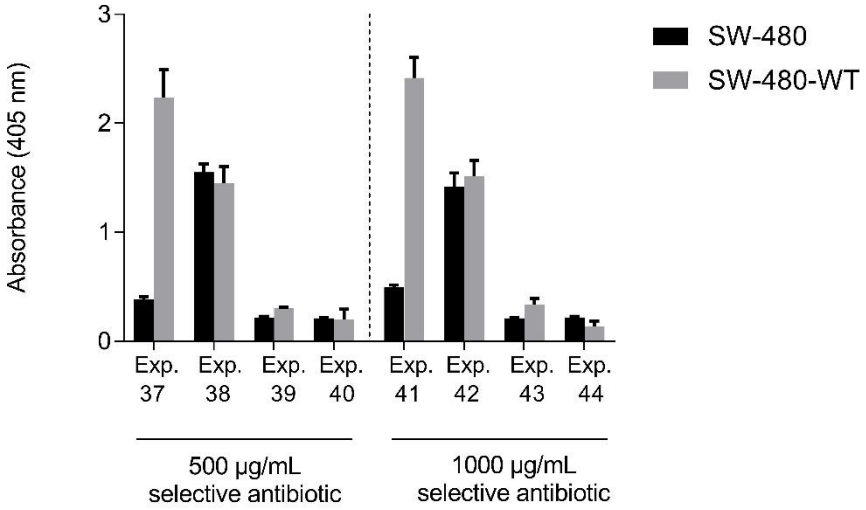


Figure 79. Direct and indirect sandwich ELISA experiment evaluating different concentrations for the selection antibiotic G-418 and different antibodies for p-50. Experiments (Exp.) 37 and 41: Bcl-3 detection, Exp. 38-42 and 43-44: p50 (see Table 16 for antibody details). General protocol in section 7.11. Mean + SEM, N=1, n=3.

There was a very clear increase in expression levels of Bcl-3 in the over-expressing cells (SW-480-WT) compared to their parental cells (SW-480), indicating successful over-expression of the protein, as demonstrated in experiments 37 and 41. The different concentration of selective antibiotic did not affect the detection of Bcl-3. At both concentration, 500 µg/mL and 1000 µg/mL, the antibodies used in experiments 39 and 40, 43 and 44 did not enhance p50 detection. At higher selective antibiotic concentration, the detected signal for p50 followed the expected results: slightly higher absorbance for the SW-480-WT (experiment 42) than in the SW-480.

Results from experiment 41 and 42 are comparable to the expected ones. However, for p50, the difference between the levels obtained from the experiments on the parental and over-expressing cell sub-lines (SW-480 and SW-480-WT) was still not detected. The difference between the absorbance of p50 in the parental and over-expressing sub-lines was still not significant (experiment 42). This may be due to three possible reasons: 1) Bcl-3 over-expression is not efficiently maintained: higher concentration of selective antibiotic, such as 1000 µg/mL, enhance the cell population to be composed of mainly cells over-expressing Bcl-3 and to kill cells which did not over-express it; 2) antibodies did not allow a reliable detection of p50; 3) in un-stimulated cells, the concentration of p50 in complex with Bcl-3 is too low; 4) as aforementioned for the optimisation in the HEK-293 cells, different steps (washing or buffer) may badly effect the outcomes of the assay. To answer at least some of these questions, different antibodies were used and LPS was used to stimulate the NF-κB pathway, and thus induce Bcl-3:p50 complex formation, as previously reported in literature.[15]

In experiment 45 and 46, lysate from cells cultured in presence of 1000 µg/mL of G-418 were probed by two different antibodies (listed in Table 17).

Table 17. Summary of different antibodies used in the indirect sandwich ELISA evaluation of p50 expression in SW-480 and SW-480-WT cells. Experiments (Exp.) 45-46 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
45	Abcam	ab31410	rabbit polyclonal	Human NF-κB p105/ p50. Synthetic non-phosphopeptide derived from Human NF-κB p105/p50 around the phosphorylation site of Serine 337	1 mg/mL
46	Abcam	ab32360	rabbit polyclonal	to NF-κB p105/ p50 aa 300-400	1.058 mg/mL

As shown in Figure 80 none of the new antibodies gives an absorbance in the same range of experiment 42, thus indicating that the alternative antibodies were not effective in detecting p50.

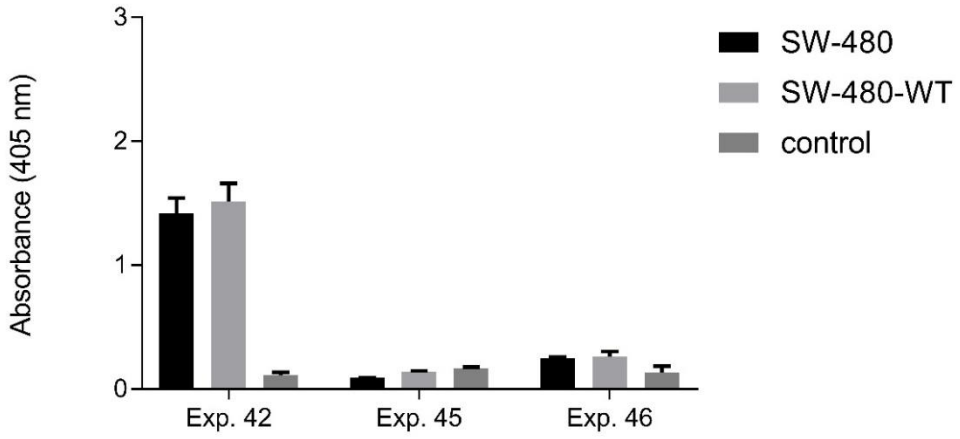


Figure 80. Indirect sandwich ELISA results with different antibodies to detect p50 in SW-480 and SW-480-WT cells. Cells were cultured in the presence of 1000 µg/mL G-418 (general protocol in section 7.11). Mean + SEM, N=1, n=3.

After this, stimulation of cells upon LPS (0.1 µg/mL, 4 hours) activation was performed.[15] For Bcl-3 detection, the new Bcl-3 antibody (23959-1-AP) was used, while for p-50 the same rabbit antibody as in experiment 42 was used (rabbit polyclonal, ab-7549, Abcam), due to its promising results in Figure 79. As reported in Figure 81, stimulation of both cell lines in presence of LPS did neither improve the absorbance nor the difference in the signal for both proteins.

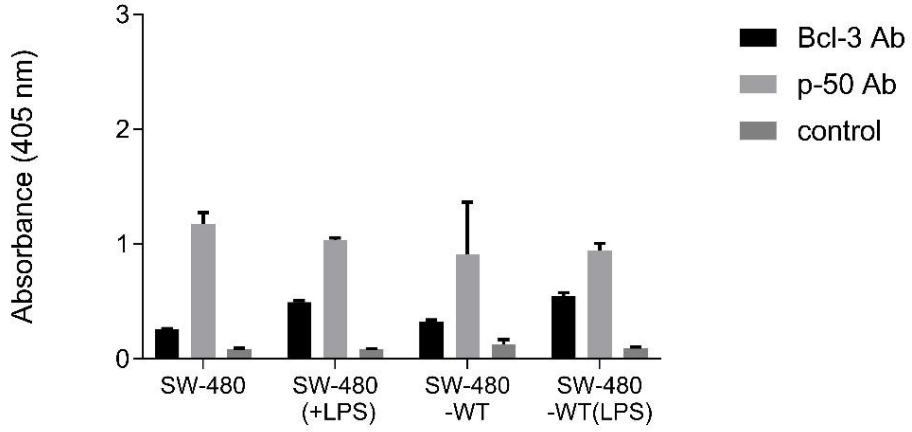


Figure 81. Direct and indirect sandwich ELISA results in SW-480 and SW-480-WT cells after LPS stimulation. Cells were cultured in the presence of 1000 µg/mL G-418 (general protocol in section 7.11). Cells were stimulated with LPS at 0.1µg/mL for 4 hours, prior to lysate preparation. Mean + SEM, N=1, n=3.

Furthermore, to investigate whether incubation time and temperature may affect the outcomes of the assay, in experiment 47, 48 and 49 overnight incubation of lysate on the plate at 4 °C (thus potentially limiting dissociation of the Bcl-3:p50 complex) was performed, while all other parameters and the used antibodies were the same as in experiment 41 (Bcl-3 detection) and 42 (p50 detection).

Table 18. Summary of different antibodies used for ELISA evaluation of Bcl-3 and p-50 expression in SW-480 and SW-480-WT cells. Experiments (Exp.) 41-42 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418, incubated one hour at 37 °C on the pre-coated ELISA plate. Experiments (Exp.) 47-49 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418, incubated overnight at 4 °C on the pre-coated ELISA plate. Experiments (Exp.) 50-52 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418: cell lysate was incubated for 24 hours at 4 °C with the primary antibody; this antigen:antibody complex was then incubated one hour on the pre-coated plate at 37 °C.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
41/47/ 50	Proteintech	23959-1-AP	rabbit polyclonal	Bcl-3 GST fusion protein	1:200
42/48/ 51	Abcam	ab-7549	rabbit polyclonal	phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
43/49/ 52	Abcam	ab-54162	rabbit polyclonal	Human NF-κB p105/ p50 (internal region of the p50 subunit)	1:200

As reported in Figure 82, overnight incubation of the lysate with capture antibody on the plate (experiment 47-49) does not improve the detection of both proteins compared to experiments 41-43 (one hour incubation).

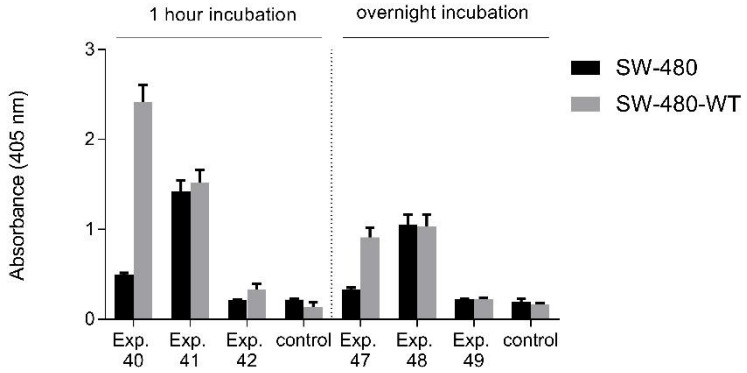


Figure 82. Sandwich ELISA, different incubation times of lysate with capture antibody on the plate. Experiments (Exp.) 41-43 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418, incubated one hour at 37 °C on the pre-coated ELISA plate. Experiments (Exp.) 47-49 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418, incubated overnight at 4 °C on the pre-coated ELISA plate to detect Bcl-3 (Exp. 47), or p50 (Exp. 48, 49) in SW-480 and SW-480-WT cells. Mean + SEM, N=1, n=3.

The usual protocol for the ELISA assay (as reported in section 7.11) entails one hour incubation at 37 °C with the capture antibody and one hour incubation of the complex (Bcl-3 protein attached on capture antibody) with the primary antibody at 25 °C. To investigate whether the incubation with the capture antibody may hide epitopes for the following reactions with the primary antibody, a different procedure was evaluated. Non-denatured lysate was incubated for 24 hours with the proper primary antibody (both Bcl-3 and p50 antibody). This conjugate was then incubated with the capture antibody on the well, thus effectively reversing the antibody binding steps. As shown in Figure 83, compared to the usual procedure (experiments 41, 42 and 43), results from the inverse procedure with 24 hour incubation of primary antibody with the lysate did not enhance detection of p50 (experiment 51, 52) and, conversely, led to a loss of signal intensity for Bcl-3 (experiment 50).

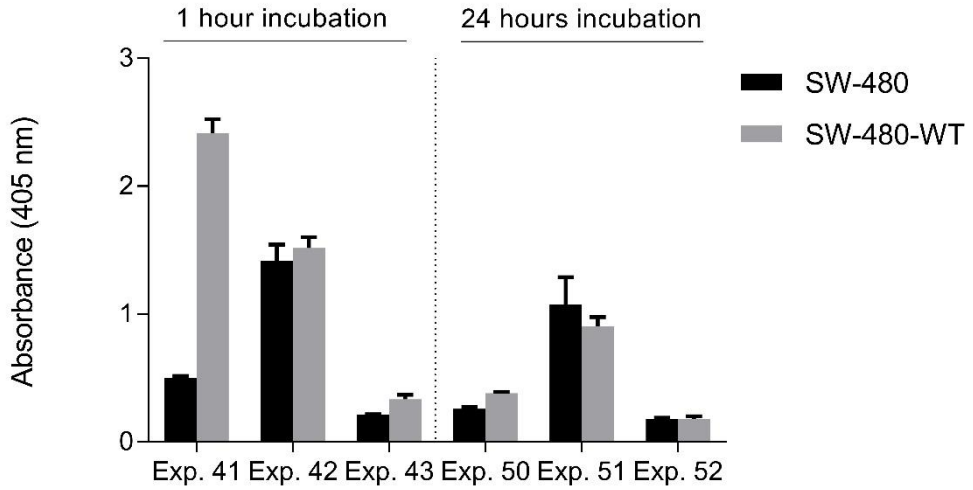


Figure 83. Different order and incubation times of lysate with primary antibody: one hour at 25°C against overnight 24°C at 4 °C to detect Bcl-3 (Exp. 49), or p50 (exp. 50, 51) in SW-480 and SW-480-WT cells. Experiments (Exp.) 41-43 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418, incubated one hour at 37 °C on the pre-coated ELISA plate. Experiments (Exp.) 49-51 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418, incubated for 24 hours at 4 °C with the primary antibody. General protocol in section 7.11. Mean + SEM, N=1, n=3.

Different dilutions for p50 antibody (ab7549) were tested to address the question if the current detection signal for p50 may be an artefact of cross reactivity among antibodies. As reported in Figure 84, the absorbance signal for p50 detection drops with decreasing antibody concentration, without any difference in the detection signal between the parental and Bcl-3 over-expressing cell line. Thus, p50 detection may not be considered reliable at all, as these results indicate that the observed signal is purely antibody-concentration dependent and could thus be classified as background.

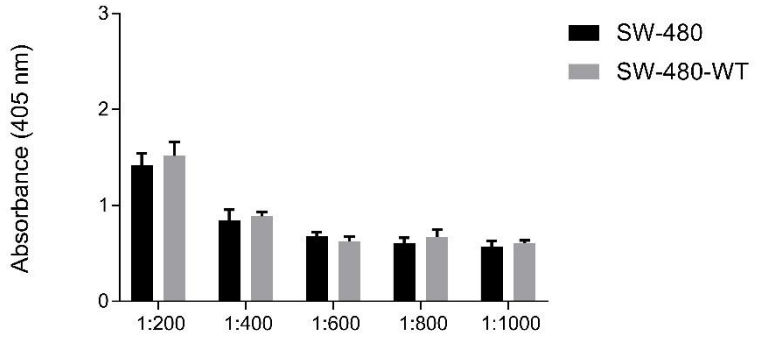


Figure 84. Different dilutions of p50 antibody. Cells cultured in the presence of 1000 µg/mL G-418. Antibody used: rabbit, polyclonal, p50 antibody (ab7549, Abcam). General protocol in section 7.11. Mean + SEM, N=1, n=3.

In experiment 53 and 54, p52 detection was investigated using two different antibodies as listed in Table 19. However, expression of p52 could not be detected, as shown in the results presented in Figure 85, without positive results.

Table 19. Summary of different antibodies used for indirect sandwich ELISA evaluation of p52 expression in SW-480 and SW-480-WT cells. Experiments (Exp.) 53-54 were performed on lysate from cells cultured in the presence of 1000 µg/mL G-418.

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
53	Santa Cruz	sc-7386	mouse monoclonal	amino acids 1-447 representing full length NF-κB p52 of human origin	1:200
54	Cell Signalling	4882s	rabbit polyclonal	endogenous levels of p100, the precursor, and p52, the mature form of NF-kappaB2	1:100

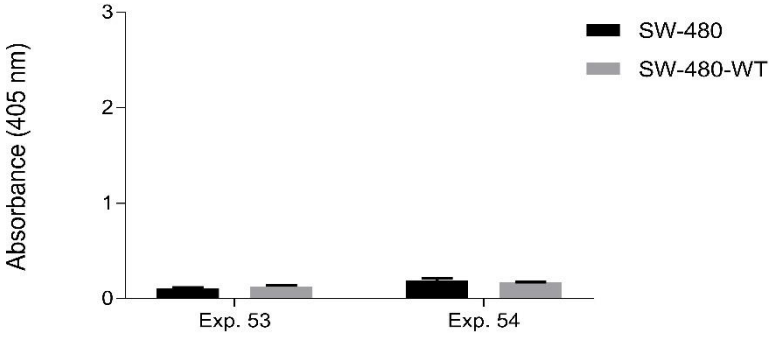


Figure 85. Indirect sandwich ELISA for different antibodies to detect p-52 in SW-480 and SW-480-WT cell lines. Cells were cultured in the presence of 1000 µg/mL G-418 (general protocol section 7.11). Mean + SEM, N=1, n=3.

For p52, a low endogenous expression level may justify the outcomes reported in Figure 85.

4.3.2.2 Conclusion

Western Blot experiments monitored Bcl-3 expression level in the tested cell lines, as a control of high endogenous Bcl-3 level the HCC1954 (further details in section 7.1) was used. As proven by Western Blot in Figure 86, after a few passages (less than 5), the transfection is not stable anymore and Bcl-3 and p50 expression levels decrease. A last experiment performed on the SW-480 in the sandwich ELISA, shown that no Bcl-3 was detected and hence no p50 as well (data non reported). These results correlate with the poor Bcl-3 detection in the cell line. Hence, it demonstrates that also this cell line is not stable in the Bcl-3 over-expression. Therefore, another cell line was tested: MDA-MB-231 and its sub-lines over-expressing Bcl-3 (MDA-MB-231-WT) will be discussed in section 4.3.3.

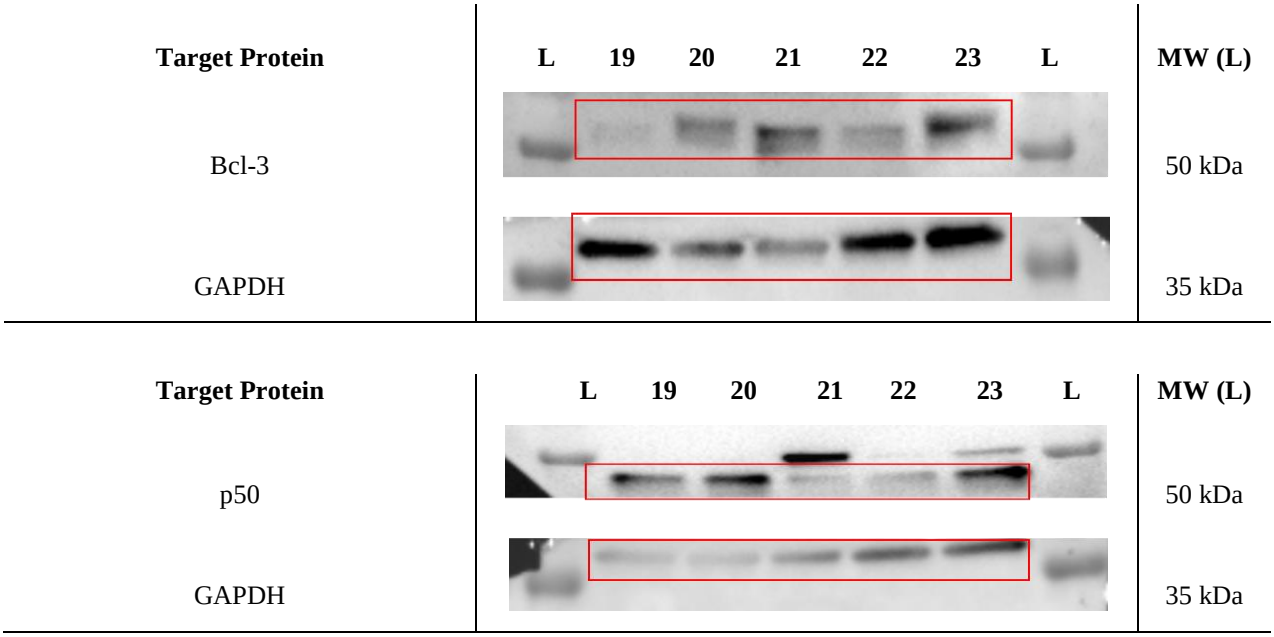


Figure 86. Western Blots for samples 19 – 23. General protocol in section 7.10. L = Ladder, 19 = SW-480, 20 = SW-480-WT, 21 = HCC1954, 22 = MDA-MB 231-P, 23 = MDA-MB-231-WT. Antibody used: rabbit polyclonal, Bcl-3 antibody (23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000).

4.3.3 MDA-MB-231 cell line

4.3.3.1 Detection of Bcl-3 and p-50 in the ELISA assay

In the effort to check if a different cell line and hence tumour microenvironment may be beneficial for the ELISA, an epithelial mammary gland/breast; derived from metastatic site cell line finally was used: the MDA-MB- 231. Its Bcl-3 overexpression level was investigated, as reported in Western Blot in Figure 86, in above section. Additionally, the panel of sub-cell lines includes the parental MDA-MB-231 which was characterised by endogenous low expression level of Bcl-3, MDA-MB-231-WT which was characterised by clear and high expression level of Bcl-3 and MDA-MB-231-ANK, characterised by high expression level of Bcl-3 and it was not able to bind p50 (further description of cell lines in section 7.1). This cell lines was used to perform ELISA experiments to detect both Bcl-3 and p50 proteins, as described below. Antibodies used to perform the ELISA run are listed in Table 20.

Table 20. Summary of different antibodies used in the sandwich ELISA in MDA-MB-231, MDA-MB-231-WT, MDA-MB-231-ANK cells. Experiments (Exp.) 55-58 were performed on lysate from cells cultured in the presence of 300 µg/mL of G-418 (section 7.2).

Exp.	Supplier	Antibody catalogue number	Species	Epitope	Working dilution
55	Proteintech	23959-1-AP	rabbit polyclonal	Bcl-3 GST fusion protein	1:200
56	Abcam	ab-7549	rabbit polyclonal	corresponding to phosphorylated Ser 337 of NF-κB p50 of human origin	1:200
57	Abcam	ab-54162	rabbit polyclonal	Recombinant fragment corresponding to Human NF-κB p105/ p50 (internal region of the p50 subunit)	1:200
58	Proteintech	14220	rabbit polyclonal	NF-κB GST fusion protein	1:200

The results are depicted in Figure 87. In general, Bcl-3 was detected at lower levels than in SW-480-WT cell lines (Figure 79, experiment 41). However, this is consistent with the expected signal for MDA-MB-231 as they are reported to have low endogenous Bcl-3 expression levels. As expected and shown in experiment 55, Bcl-3 over-expressing MDA-MB-231-WT cells, as well as MDA-MB-

231-ANK over-expressing sub-line, which is mutated not to bind p50 (further details in section 7.1) did show a higher abundance of Bcl-3 than non-transfected MDA-MB-231 cells.

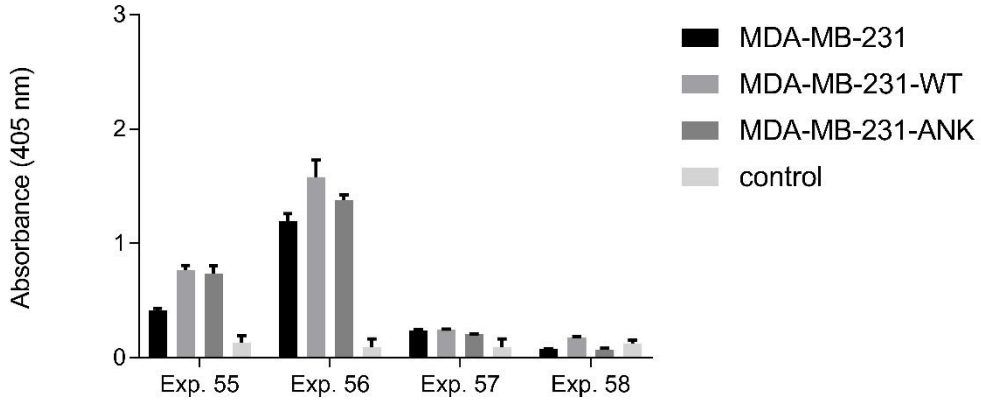


Figure 87. Direct and indirect sandwich ELISA to detect Bcl-3 and p-50 in MDA-MB-231, MDA-MB-231-WT, MDA-MB-231-ANK mutant. Cells were cultured in the presence of 300 µg/mL of G-418 (general protocol in section 7.11). Mean + SEM, N=1, n=3.

For p50 detection, experiment 56 highlighted that all the three sub cell lines which should give a different detection signal for p50, indeed gave the same results with no significant difference among the three of them. The MDA-MB-231-ANK mutant, which did not bind p50 (section 7.1 for more details) gave an absorbance in the same range of the MDA-MB-231 cells which should have given a significant lower value of absorbance and of the MDA-MB-231-WT which should have given a higher signal for p50.

Different conclusions may explains these results. First, p50 antibody cross reacts with lysate and capture antibody, hence the absorbance could be higher than the actual and real one. Another possible explanation may be that if the signal for p50 is reliable in all of the three cell lines (due to the consistent signal detected), then the over-expression of Bcl-3 was not efficient due to the tiny difference in the absorbance detected for p50. On the other side, if the actual signal for p50 is reliable, the low difference may be due to the lack of cell stimulation to activate the formation of the Bcl-3:p50 complex. Indeed, as reported in section 1.6.3.3, stimulation with different cytokines activates the cascade of pathway 3 (Bcl-3 pathway) which consequently leads to processing of p105 in p50, formation of the homodimers and translocation in the nucleus where they bind Bcl-3.

A last explanation may be that the complex is formed but since no more information are available in the literature about the stoichiometry of the complex and the exclusive presence of p50 (indeed, the

complex may be formed by Bcl-3/p50/p52), the low absorbance difference among the three cell lines is due to low quantity presence of p50 in the complex itself.

Finally, Western Blot analysis, reported in Figure 88, showed that also this cell line lost Bcl-3 over-expression and it was not stable, justifying the last results for the ELISA where no Bcl-3 was detected (data not reported).

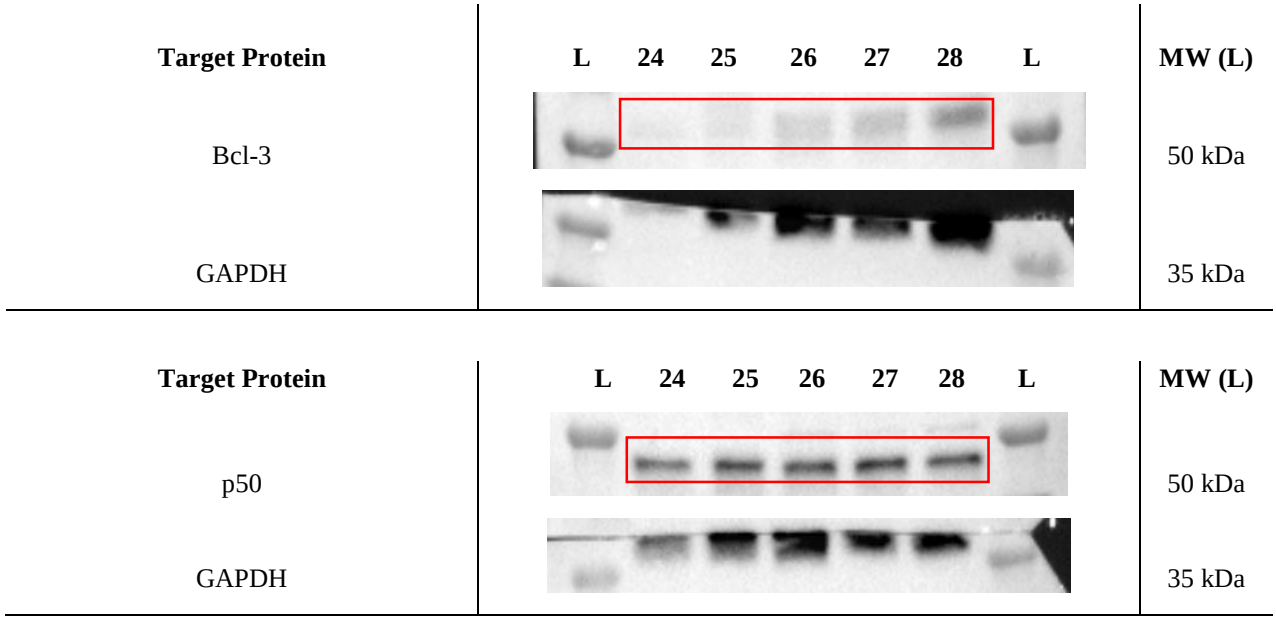


Figure 88. Western Blots for samples 24 – 28. L = ladder, 24 = HEK-293, 25 = HEK-WT-7, 26 = HCC1954, 27 = MDA-MB-231, 28 = MDA-MB-231-WT. General protocol in section 7.10. Antibody used: rabbit polyclonal, Bcl-3 antibody (23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000). Passage 5.

4.3.4 Conclusions

Although the ELISA is one of the most used technique to detect the protein-protein interactions, many limitations have hampered its successful optimisation in this project.

Initially, the HEK-293 cell line and its sub-cell line were used. Although, as demonstrated by Western Blot after few passages the expression was lost (Western Bot at passage 1 in Figure 62, Western Blot at passage 5 in Figure 88). On this cell lines, many parameters were checked to investigate whether they may have a detrimental influence on the results. Crucial parameters, such as the detergent used to dilute lysate or antibody, washing solutions, blocking solutions and incubation times were investigated. None of them improves the detected absorbance for Bcl-3, conversely few of them increases the background signal detected.

Biochemical parameter such as the stimulation of complex formation in presence of cytokine (TNF-α) was evaluated: however, a decrease in the detected absorbance was observed for Bcl-3 and no improvement in the p50 detection was achieved.

Finally, a new promising antibody demonstrated to be useful in the Bcl-3 detection, as proved in Figure 70, experiment 20.

For p50, many antibodies were tested, but none of them resulted to efficiently detect it. This may be due to several reasons. For instance, the tested antibodies were not efficient in detecting p50: more antibodies may be evaluated. On the other hand, the number and the volume of the washes (parameters which were not investigated before) may disrupt the Bcl-3/p50 binding, suggesting that the actual absence of an absorbance signal different from the background is not due to the limited availability of antibody, but rather to the lack of p50 in the complex. To check these specific parameters, the use of recombinant proteins for Bcl-3 and p50 and moreover the formation of the complex using the recombinant proteins may alleviate these issues. Additional experiments for the ELISA based on recombinant proteins were performed, but due to the limitation of information about stoichiometry of the complex and the absence of a commercially available full length recombinant protein p50, these experiments exhibited no improvements in specific signals (data not reported).

Due to the lack of stable over-expression of Bcl-3 in HEK-293 cells, a more stable transfected cell line was used: SW-480 and its wild type sub line. Because the aforementioned parameters such as buffer for the dilution of antibody or lysate, washing or blocking solutions and stopping solutions did not improve the previous results in initial experiments on HEK-293 cells, these parameters were not investigated on this cell line. In the SW-480, SW-480-WT alternative parameters were evaluated: different antibodies, LPS stimulation and a reverse order in the general protocol of the ELISA. None of the tested parameters affected positively the results. However, a better signal for p50 was achieved with one of the tested antibodies (14220-1AP). Although the signal detected for both Bcl-3 and p50 was greatly improved, the difference of the signal between the parental and the wild type cell line was still tight. Several explanations may be presented to justify this. However, the most likely explanation based on existing evidence is that the detectable p50 signal was due to cross-reactivity of the primary antibody with either the capture antibody or the plate, while Bcl-3:p50 complex formation was suboptimal in the incubation/wash conditions employed to date.

Secondly, the actual transfection was not so efficient. The main hypothesis is based on two milestones: Bcl-3 is found to be over-expressed in several solid tumours (included breast cancer) and in presence of particular stimuli, p50 level in the nuclear is increased and thus more of it may bind

Bcl-3 (to activate the metastatic progression of the genes). Hence, a sharp difference in the actual absorbance signal for p50 between the two different cell lines is theoretically expected: SW-480, with a low endogenous expression level of Bcl-3, does not have any Bcl-3:p50 complex and hence the expected absorbance for both proteins should be in the same range of the control. Conversely, the SW-480-WT was shown to express higher amount of Bcl-3, thus of Bcl-3:p50 complex, hence the absorbance detected should be higher and significantly different from the ones detected in the parental line.

Finally, the metastatic breast cancer cell line MDA-MB-231 was used as an alternative model in the event that a different cellular context may improve complex formation in our conditions. Additionally, for this specific cell line was possible to adopt an additional control sample (the ANK-Bcl-3 binding mutant, which is not able to bind p50). The antibodies for Bcl-3 and p50 detection, coming from the optimisation study on the previous cell lines, were used and they proved to be suitable in this cell line. The MDA-MB-231 cell line and its sub-cell lines are interesting due to the possibility of using the ANK mutant, which represents a further control in the experiment. Hence more experiments will confirm the results shown in Figure 87.

4.4 Immunoprecipitation and co-Immunoprecipitation

To prove the Bcl3/p50 interaction, immunoprecipitation (IP) was used as effective tool due to its high sensitivity. This technique allows the isolation of target proteins out of complex sample mixtures using antibodies.[19] The general classical procedure, depicted in Figure 89, involves the following steps: 1) extraction of target proteins from cell lysate in an appropriate lysis buffer; 2) incubation of the cell lysate with a specific antibody to allow formation of the immune complex; 3) precipitation of the complex on a solid phase support (antigen-antibody complex is bound to Protein A or G beads). Centrifugation of the beads provides a pellet made up of the captured immune complex. The captured protein (pellet) is then eluted from the immunocomplex by exposure to denaturing or low-pH conditions. Once the target proteins are released, they may be analysed via one-dimensional or two-dimensional gel electrophoresis and immunoblotting.[20]

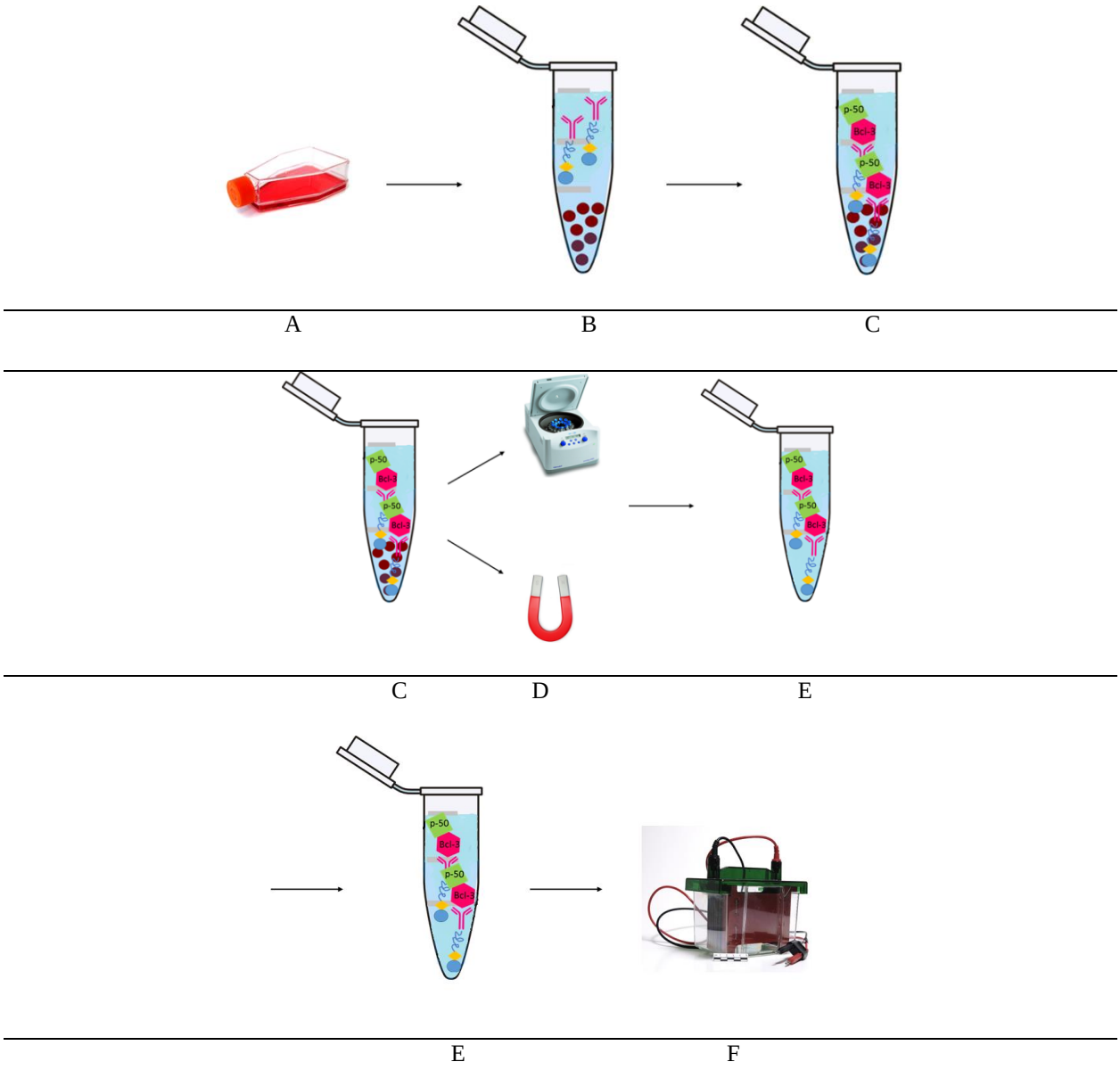


Figure 89. Schematic representation of steps A-G of immunoprecipitation: A) Lysis of cultured cells and preparation of samples; B) incubation of cell lysate with solid support conjugated antibody; C) formation of the immunocomplex: target protein conjugated with the antibody on the solid support; D) precipitation of imunocomplex by use of centrifuge (classical IP) or magnet (modern IP); E) elution of the immunocomplex from the solid support; F) analysis by Western Blots.

However, the classical IP methodology has several drawbacks: use of large quantities of costly IP antibodies, sample loss when the supernatant is removed from a small pellet and general low efficiency of the technique due to the binding of the antibody to the solid support. Additional disadvantages are specific for the application of the technique to the Bcl-3:p50 complex. For instance, the last step of the general procedure involves the release of both antibody and captured protein. The molecular weight of the targeted proteins and the molecular weight of heavy chains of the antibody are the same (around 50 kDa). Thus, the traditional IP method may be hampered by contamination of

heavy chains from the antibodies as the bands of the target protein will co-migrate during an SDS-PAGE run.

Due to the small molecular weight of both target proteins, a modified IP technique was used, which entails the use of magnetic beads as solid phase support instead of protein A/G. The use of magnetic beads improves both selectivity and yield of the reaction between antibody and cell lysate; additionally, it allows for a better separation of the beads itself from the complex sample mixture. Hence, the magnetic beads allow for a cleaner precipitation of target proteins and reduce loss of sample due to pipetting errors. Moreover, the magnetic beads have a faster rate of protein binding compared to the use of agarose beads.[21] Magnetic beads have a higher binding capacity for the antibody attached on them, although the amount of antibody required to saturate the bead is higher than the one needed for agarose beads, which can impact the overall cost of the technique as a single attempt, but it is cheaper in a scale up process where more IPs are required to be performed.[20] Additionally, the agarose beads have a lower binding capacity, but also non-specific binding is higher than in magnetic beads.

The general principle is to use anti-Flag conjugated magnetic beads (murine anti flag M2 monoclonal antibody attached to super-paramagnetic iron, 4% agarose beads) to pull down FLAG-tag-Bcl-3. These beads are commercially available. Conversely, there are no commercially available conjugated beads for p50. This lack provides the opportunity to investigate the chemical reaction for the production of p50 antibody immobilised on magnetic beads. For p50 protein the strategy involves the conjugation of p50 antibody with so named "Dynabeads protein G conjugated". Dynabeads are super-paramagnetic spherical polymer particles with a defined surface for the coupling of biomolecules.[22] Advantages regarding their use involve low background signal, high yield, specificity and low non-specific binding.

To further increase the sensitivity and the efficiency in the conjugation of the antibody with the beads, it was necessary to introduce a further step: the introduction of a cross linker. The oriented affinity method allows the orientation of the capture antibody using its Fc region (Figure 90). In brief, to covalently attach the antibody to the beads, dimethyl pimelimidate dehydrate (DMP) was used as a common homo bi-functional cross linker. The reactive imidoester moiety at both ends of the DMP interact with amino groups/primary amines which are located on lysine residues and on the *N*-terminus of each polypeptide chain which are distributed over the entire antibody.

Figure 90. Oriented, cross-linked antibody for IP uses. The use of the crosslinker (DMP) enhances the orientation of the antibody towards the target protein.

The absence of the cross-linker may decrease the number of epitopes (as depicted in Figure 91) involved in the immunoprecipitation reaction of the protein with the antibody, leading to a loss of sample and, thus, in the end, to the lack of the specific band in the gel of the Western Blot analysis.

Figure 91. Direct antibody immobilisation IP, without cross-linker. The epitope of the antibody may be masked due to their untidy orientation.

The reaction between the imidoester crosslinkers and primary amines form amidine bonds. To ensure specificity for primary amines, imidoester reactions must be performed in amine-free, alkaline conditions (pH 10).[23]

To sum up, general steps involved in the IP are listed below:

1) Preparation of lysate in non-denaturing conditions to preserve the protein-protein interaction.
2) In parallel incubation with magnetic beads (anti-flag and p50 conjugated magnetic beads).
3) Application of magnet to pull down the immune complex.
4) Washes.
5) Denaturation of immune complex and preparation samples for Western Blot.

6) Cross Western Blots: on samples that were immunoprecipitated using anti-FLAG conjugated beads, a Western Blot was performed using p-50 antibody in the first run and Bcl-3 in the second run after membrane stripping. On samples that were immunoprecipitated using p-50 conjugated magnetic beads, Western Blot was performed first using Bcl-3 antibody in the first run and p50 in the second run after membrane stripping.

4.4.1 Immunoprecipitation and co-immunoprecipitation preliminary results

Prior to their use in the IP and co-IP experiments, various cell lines were tested by Western Blot to ensure Bcl-3 and p-50 expression, as reported in Figure 92. SW-480 cells were used as a control for low endogenous Bcl-3 expression level, conversely SW-480-WT and HCC1954 were used as a control for high Bcl-3 expression, as proved by the intensity of the detected band. As a result, the MDA-MB-231 (sample 32) shown a light band, indicating the low endogenous Bcl-3 expression level. The MDA-MB-231-WT (sample 33) shown the intense band for Bcl-3 at the proper molecular weight. The same cells were tested for p50: only SW-480, SW-480-WT and MDA-MB-231-WT exhibited a detectable p50 band.

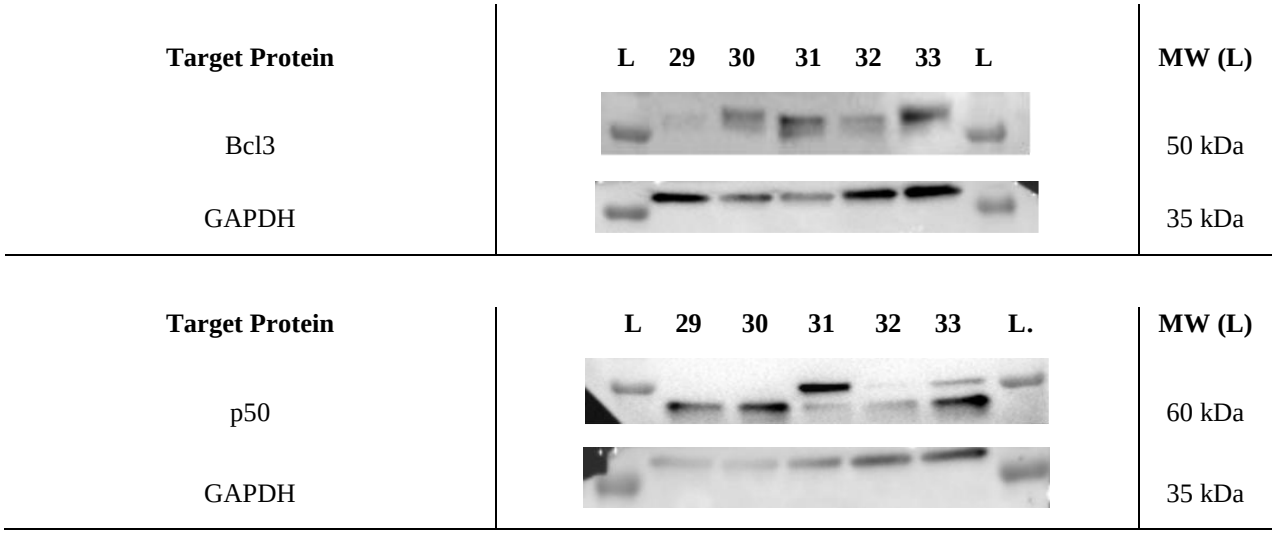


Figure 92. Western blots to select highly Bcl-3 over-expressing cell lines (sample 29- 33). General protocol in section 7.10, 7.12. L = ladder, 29 = SW-480, 30 = SW-480-WT, 31 = HCC1954, 32 = MDA-MB-231, 33 = MDA-MB-231-WT. Antibody used: rabbit polyclonal, Bcl-3 antibody (23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000).

The promising preliminary results for the IP and the co-IP are displayed in Figure 93 and Figure 94. Briefly, MDA-MB-231 and the wild type cell line were used as further control to detect the Bcl-3 and p50 bands in the Western Blot analysis. Sample 36 is the parental cell line, MDA-MB-231, which

was immunoprecipitated using the anti-FLAG conjugated magnetic beads and in the run of the Western Blot, a rabbit polyclonal antibody against p-50 was used. Sample 37 is the cell line MDA-MB-231-WT which was immunoprecipitated using the anti-FLAG conjugated magnetic beads and in the run of the Western Blot, a rabbit polyclonal antibody against p50 was used. The p50 western blot (Figure 93.a) demonstrated a small increase in p50 in the IP of Bcl-3 over-expressing cells, relative to the western blot of Bcl-3 in the sample samples, suggesting the presence of an increased proportion of the Bcl-3:p50 complex in sample 37. The same membrane was stripped and a second Western Blots using the Bcl-3 primary antibody was performed to confirm the presence of Bcl-3 (Figure 93.b).

As shown in Figure 93, the relative proportion of p50 and Bcl-3 in total cell lysates of MDA-MB-231 parental and Bcl-3 over-expressing lines were indistinguishable, further supporting the conclusion that Bcl-3:p50 complex was more prevalent in the over-expressing cell line.

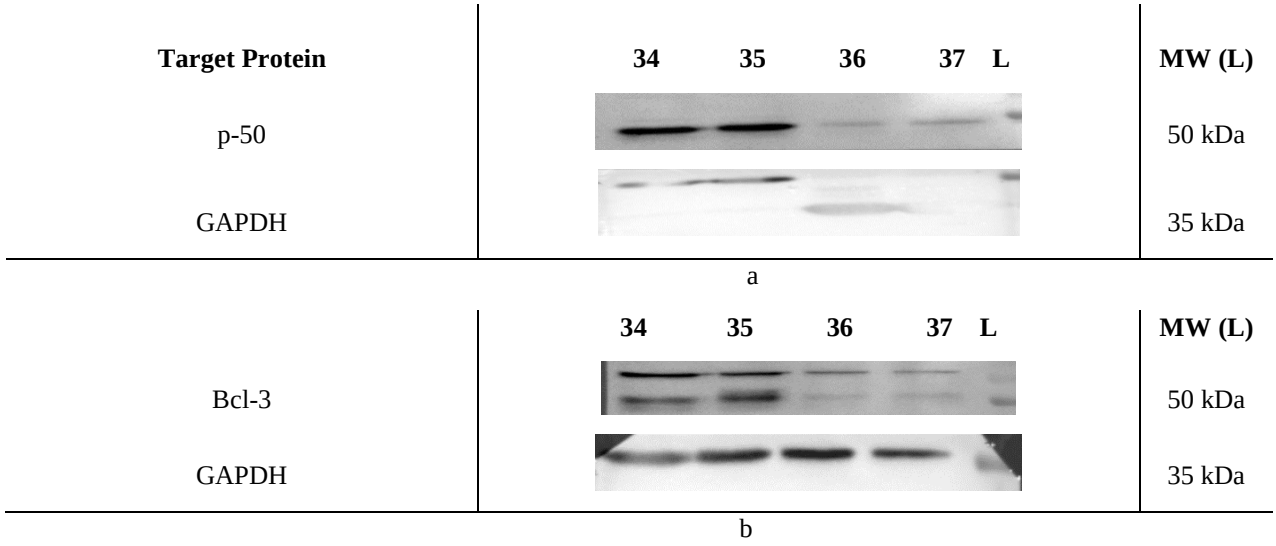


Figure 93. Western Blots for IP results (sample 34 – 37). General protocol in section 7.10, 7.12. Cells are pulled down with ANTI-FLAG beads, membrane were incubated with primary antibody for p50 (a) and Bcl-3 (b). 34 = MDA-MB-231, 35 = MDA-MB-231-WT, 36 = IP-antiFLAG-MDA-MB-231, 37 = IP-antiFLAG-MDA-MB-231-WT, L = ladder. Antibody used: rabbit polyclonal, Bcl-3 antibody (23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000).

Further evidence of the presence for the actual formation of the complex is presented in Figure 94, where the results of the co-IP (on the "inverse" IP) are represented. Sample 38 is the parental cell line, MDA-MB-231, which was immunoprecipitated using the p50 conjugated dynabeads and in the run of the Western Blot, a rabbit polyclonal antibody against Bcl-3 was used. Sample 39 is the cell line MDA-MB-231-WT which was immunoprecipitated using the p50 conjugated dynabeads and in the run of the Western Blot, a rabbit polyclonal antibody against Bcl-3 was used. The presence of the

intense band in correspondence of 55 kDa for all the samples, demonstrate the presence of Bcl-3 in complex with the initially "pulled down" p-50.

A second Western Blot with the p-50 primary antibody on the same, stripped, membrane shows a labile band in correspondence of p-50 molecular weight (Figure 94).

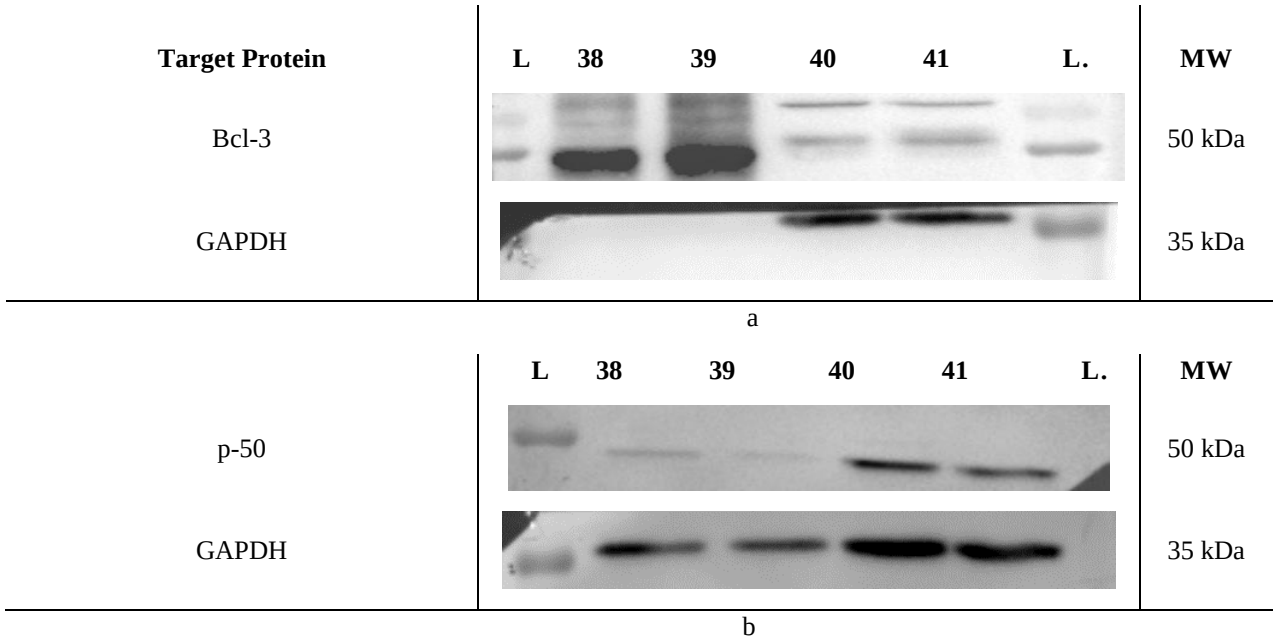


Figure 94. Western Blots for co-IP results (sample 38 – 41). General protocol 7.10, 7.12. L= ladder, 38 = IP-dyna-231, 39 = IP-dyne-231-W, 40 = MDA-MB-231, 41 = MDA-MB-231-WT. Antibody used: rabbit polyclonal, Bcl-3 antibody (a, 23959-1-AP, Proteintech, 1:1000); rabbit, polyclonal p50 antibody (b, 14220-1-AP, Proteintech, 1:1000); mouse, monoclonal GAPDH antibody (sc-32233, Santa Cruz, 1:5000).

The disparity between the intensity of Bcl-3 and p50 bands in western blots of IP-p50 samples is a concern for the specificity of this pull-down experiment. It will therefore be necessary to repeat the p50 pull down experiment, also including a control for the heavy chain of antibody to make sure that the band observed in the region of interest is specific for the target proteins.

The use of a general anti-rabbit immunoglobulin as a blocking solution to definitely address the questions about possible detection of the heavy or light antibody chains rather than the actual p50 protein will be performed to ensure reliability of preliminary results in the future.

In conclusion, these preliminary results encourage future exploration of this technique to monitor the Bcl-3/p50 interactions within cells and also to show the on-site effect of drugs against the protein complex.

4.5 Colony forming assay

The colony forming assay (CFA) or clonogenic assay was chosen as a predictive screening assay to identify active compounds as it is a traditional method for medium to high throughput *in vitro* evaluation of drug effects on clonal expansion of tumour cells.[16] It is an *in vitro* system designed to monitor the ability of each cancer cell in the tumorigenic population to undergo unlimited division and to produce a viable colony in the presence of either a vehicle (DMSO, in equimolar concentration with tested compounds) or drugs, regardless of the mechanism of action of the drug.[17] There are several advantages with regards to the use of this assay: good reproducibility, different cell lines and different concentration to test over a relatively short period of time, relatively inexpensive, and, finally, simplicity.[18]

The HCC1954 cell line was selected to perform the colony forming assay because of several features: it is a triple negative metastatic breast cancer cell line with high endogenous Bcl-3 level, which forms round shaped and well-defined colonies. The number of cells seeded that are being seeded for the colony forming assay was previously reported.[18]

It is well known that the incubation time for the assay (i.e. the time from seeding to the endpoint in which the cells are growing in the presence of vehicle or drug) must be equivalent to six cell divisions. Moreover, the cells must form colonies which consist of at least 50 cells. The diameter of the colonies at the end point should be around 240 µm.[17] Experimentally, it was found that an endpoint of 12-14 days is required to achieve the 240 µm diameter of cell colony with the tested cell line (details in section 7.1, 7.2, 7.13).

Compounds were tested using different cell lines (description of the cell lines are reported in section 7.1). On-target action of the drugs was investigated by comparing the activity of compounds in cell lines that either do or do not over-express Bcl-3 (results in section 5.2).

Finally, in the drug response study, concentration response curves were generated for the most active compounds identified by this predictive screening (results in section 5.2).

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Section 5:

RESULTS AND DISCUSSION

5.1 Cell titer blue assay results

First, newly synthesised compounds were evaluated for cytotoxicity *in vitro* at two different concentrations (10 µM and 1 µM) on both tumourigenic (MDA-MB-231) and non-tumourigenic (HEK-293) cell lines. Briefly, cells were cultured in adherent growth conditions and cell viability was determined after 24 hours incubation with Cell Titer Blue viability assay. Relative intensity of fluorescence (reported as percentage of relative fluorescence unit, % RFU) was normalised against RFU of the negative control (0.1% (v/v) and 0.01% (v/v) DMSO = 100%). The detailed protocol of the Cell Titer Blue assay is reported in section 7.7. Compound **23** was used as reference compound and additional negative control: its cytotoxic profile was established previously.[1] Carboplatin was used as positive control.

Statistical analysis was performed with Prism 7.1 software (GraphPad Software, Inc, La Jolla, CA):

1) Anova, Dunnet's multiple comparison and Tukey's multiple comparison: to compare each compound against the others and each compound against positive control, respectively. All test compounds are significantly ($p < 0.0001$) compared to positive control (* not reported).
2) One sample t-test: to compare compounds against negative control (100%).

Significances are given as ns ($p< 0.12$), ∗ ($p < 0.033$), ∗∗ ($p < 0.002$), and ∗∗∗ ($p < 0.001$) according to the New Journal of England Medicine.

Figure 95 - Figure 103 show results for tumourigenic cell line MDA-MB-231 and Figure 104 shows results for the non-tumorigenic cell line HEK-293. Additionally, Figure 95 -Figure 102 report results of analogues of compound **23**. None of the compounds tested was found to be cytotoxic compared to positive control, over 24 hours incubation in the used tumorigenic cell line (MDA-MB-231).

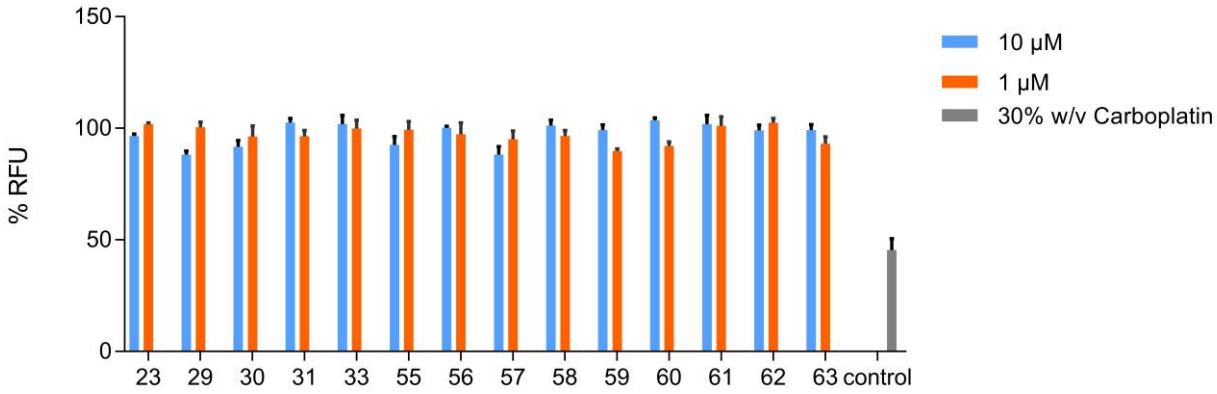


Figure 95**.** Cell titer blue results for compounds **23**, **29**-**31**, **33**, **55**-**63** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

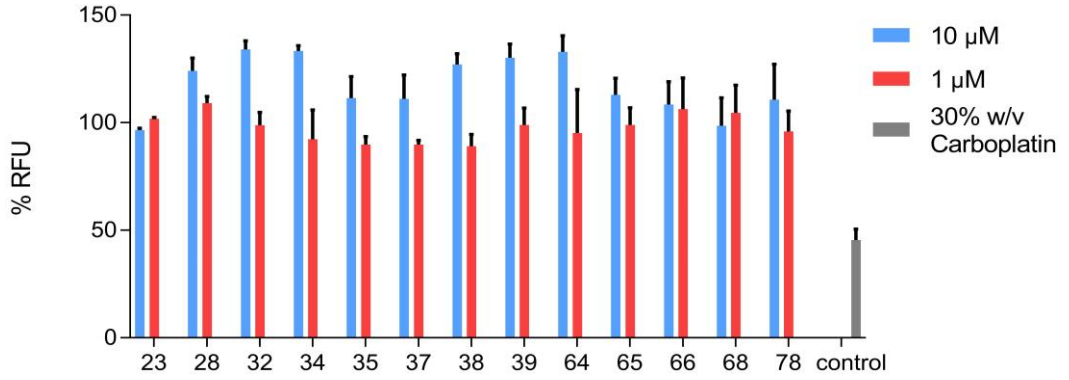


Figure 96. Cell titer blue results for compounds **23**, **28**, **32**, **34-35**, **37-39**, **64-66**, **68**, **78** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

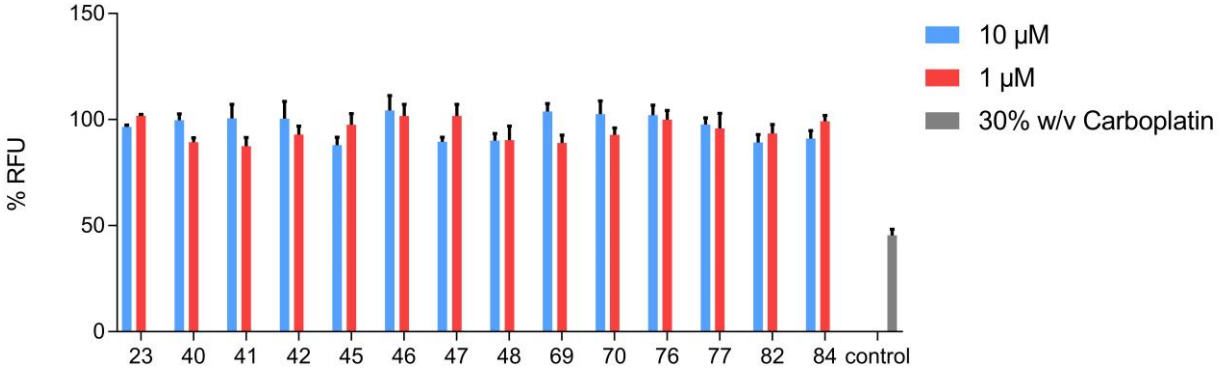


Figure 97. Cell titer blue results for compounds **23**, **40-42**, **45-48**, **69-70**, **76-77**, **82**, **84** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

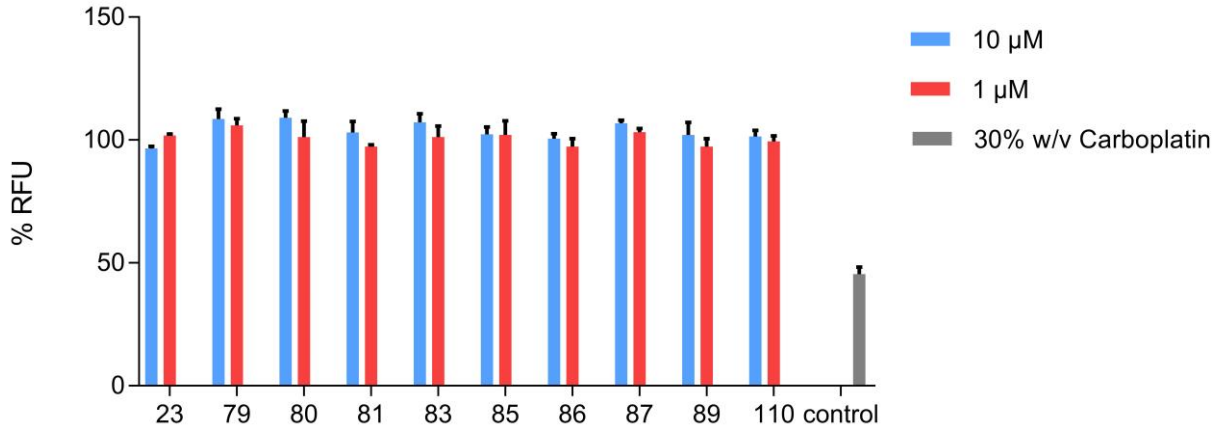


Figure 98. Cell titer blue results for compounds **23**, **79-81**, **83**, **85-87**, **89**, **110** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

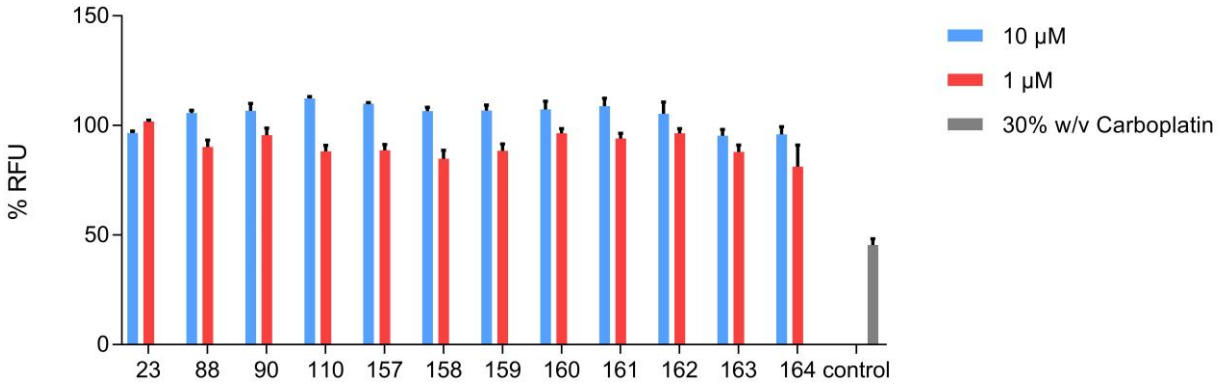


Figure 99. Cell titer blue results for compounds **23**, **88**, **90**, **110**, **157**-**164** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

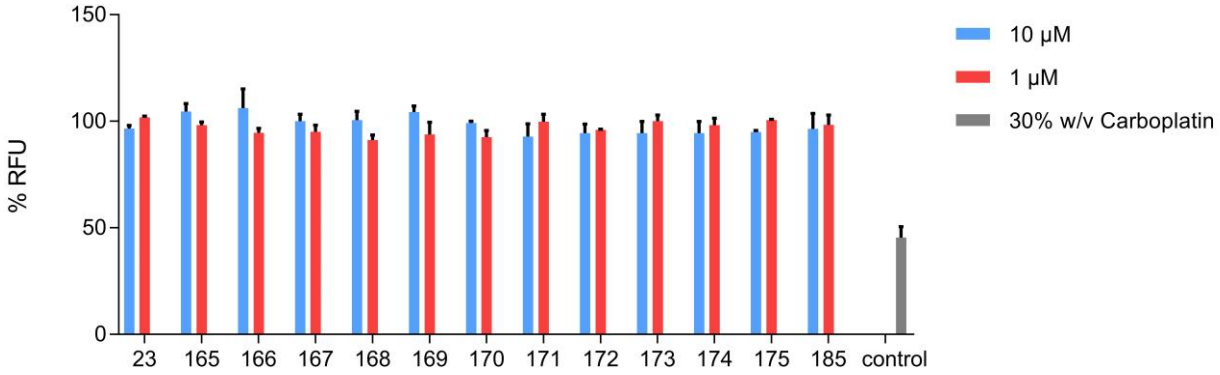


Figure 100. Cell titer blue results for compounds **23**, **165**-**175**, **185** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

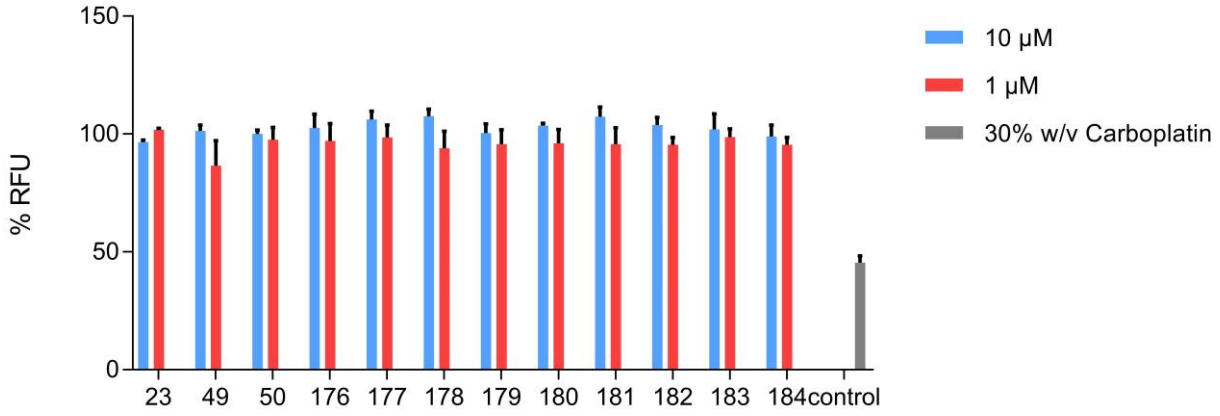


Figure 101. Cell titer blue results for compounds **23**, **49**-**50**, **176**-**184** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

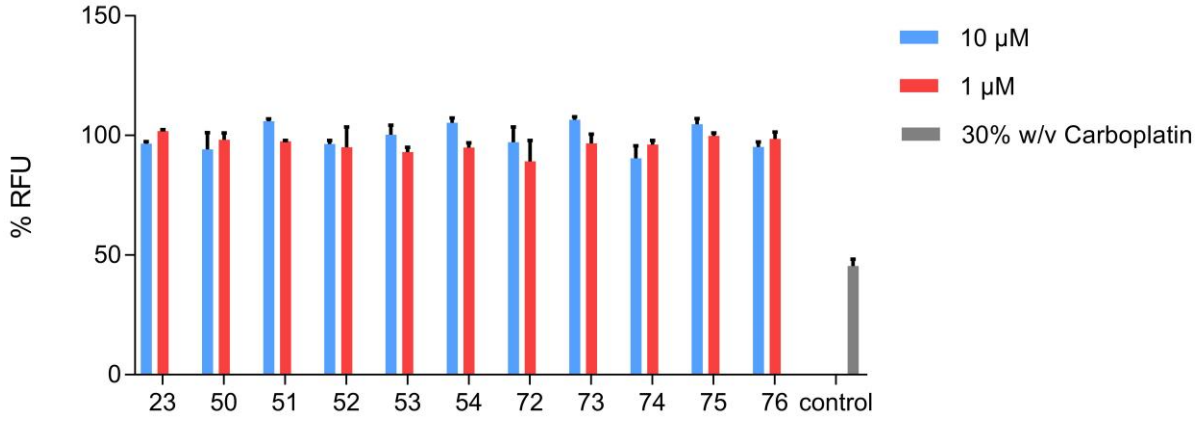


Figure 102. Cell titer blue results for compounds **23**, **50**-**54**, **72**-**76** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

Compounds from different scaffolds were tested to establish whether they may have a cytotoxic profile when tumourigenic MDA-MB-231 cell line was cultured in presence of compounds for 24 hours prior to cell titer blue reagent treatment at both 10 µM and 1 µM.

Compounds **222** and **225** are a "closed" analogue of compound **31** and **23**, compounds **191**-**210** are analogues of compound **20**. Compounds **212**-**217** are analogues of compound **26** and compound **219** is an analogue of compound **27**. As reported in Figure 103, none of compounds tested from different scaffolds have a cytotoxic profile compared to positive control and to negative control.

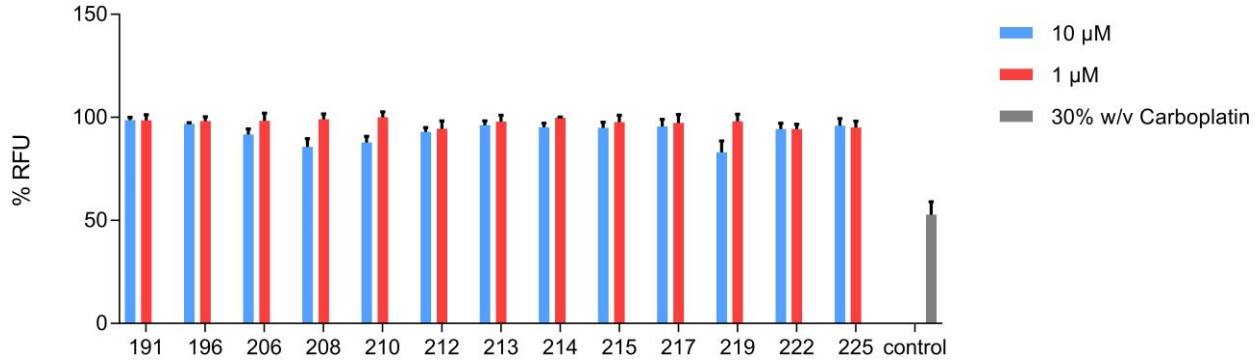


Figure 103. Cell titer blue results for compounds **191**, **196**, **206**, **208**, **210**, **212 - 215**, **217**, **219**, **222**, **225** in MDA-MB-231 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the MDA-MB-231 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

New analogues from different scaffold were also tested on non tumourigenic cell line (HEK-293): as shown in Figure 104, none of the test compounds results to be cytotoxic compared to positive control.

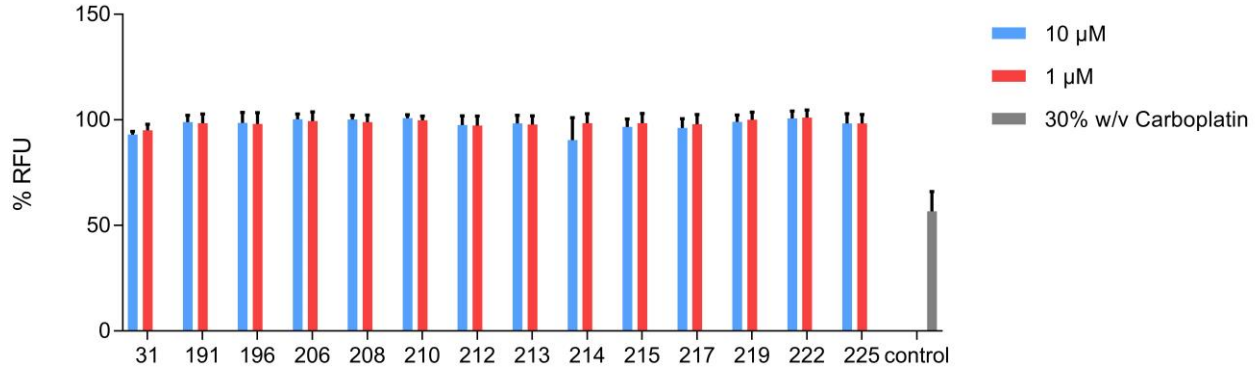


Figure 104. Cell titer blue results for compounds **31**, **191**, **196**, **206**, **208**, **210**, **212** - **215**, **217**, **219**, **222**, **225** in HEK-293 cell line. Compounds were incubated at 10 µM and 1 µM for 24hours in the HEK-293 cells. Cell titer blue reagent was then incubated for 2 hours. Absorbance was measured using Clariostar Luminescence plate reader. % RFU= % relative fluorescence unity. Mean + SEM, N=3 and n=3 (for compounds), n=6 (for control).

5.2 Colony forming assay results

In brief, cells were seeded at a relatively low dilution (200 cells/mL) in a 12-well plate. After one hour, compounds were added at a concentration of 10 µM. Media was changed every 6 days and the 10 µM drug treatment was also replaced. The number of colonies was normalised against the number of seeded cells (200 cells/mL). Further details are reported in section 7.13.

All values obtained in cell culture experiments were normalised against negative control (1% v/v and 0.1% v/v DMSO = 100%).

Statistical analysis and concentration-response curve were calculated with Prism 7.1 software (GraphPad Software, Inc, La Jolla, CA):

For normal distributed data (as assessed by D'Agostino & Pearson and Shpiro-Wilk normality tests), two statistical tests were performed:

1) Anova, Dunnet's multiple comparisons test: to compare each compound against reference compound.
2) One sample T-test: to compare each compound against the negative control (100%).

For not normally distributed data, two statistical tests have been performed:

1) Normality test: a) D'agostino & Pearson;
   b) Shapiro-Wilk.

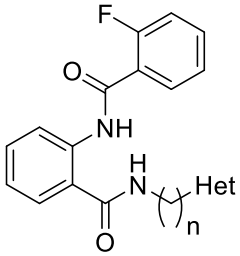
2) Kruskall-Wallis non parametric test: to compare each compound against reference compounds.

Significances are given as ns ($p < 0.12$), $*$ ($p < 0.033$), $**$ ($p < 0.002$), and $***$ ($p < 0.001$) according to the New Journal of England Medicine.
Asterisks in black refer to significance against negative control. Asterisks in blue refer to significance among compounds.

5.2.1 Colony forming assay results: analogues of compound 23

Several analogues of compound **23** were tested in the colony forming assay (CFA) on HCC1954 cell line (high endogenous Bcl-3 expression level, further details in section 7.1, 7.13) to identify putative active compounds, with improved activity profile compared to reference compound **23**.
Firstly, the effect of the replacement of the morpholine ring in compound **23** with different heterocycles and different length for the carbon linker chain were evaluated. Test compounds and their substitutions are listed in Table Table 21. Results are reported in Figure 105.

Table 21. Substitutions of test compounds in Figure 105.



Heterocycle	Morpholine		Phenyl		*N*-methyl piperazine		Piperazine
Carbon linker chain length (n)	n=2	n=3	n=2	n=3	n=2	n=3	n=2
Compound	**23**	**119**	**63**	**120**	**183**	**93**	**160**

As shown in Figure 105 substitution of the morpholine ring in compound **23** with phenyl (compound **63**) or *N*-methyl piperazine (**183**) does not improve activity. However, replacement with piperazine (**160**) leads to a further 29% reduction in the colony formation compared to compound **23**. Elongation of the carbon linker chain in the analogue with the morpholine ring (compound **119**) does not enhance activity of compound **23**. Conversely, introduction of phenyl or *N*-methyl piperazine rings in the elongated analogues (three carbon linker chain) **120** and **93** respectively,

significantly improve activity respectively with a 17% and 35% decrease in the percentage of formed colonies compared to vehicle control.

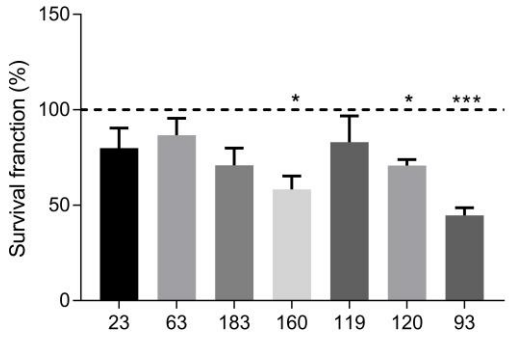
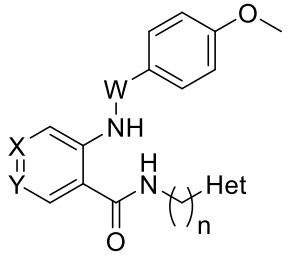


Figure 105. CFA results for compounds **23**, **63**, **183**, **160**, **119**, **120**, **93**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

In previous biological results (as mentioned in section 1.8), compound **186** was identified as a promising candidate in a small library of analogues. Hence, introduction of the electron donating group $–OCH_3$ in 4 position on the phenyl ring was further evaluated with the same approach as above: compounds with different heterocyclic/carbocyclic rings and different length for the carbon linker chain as well as the sulfonyl isoster were tested. The different substitutions are listed in Table 22, results are presented in Figure 106.

Table 22. Substitutions of test compounds in Figure 106.



Heterocycle	Morpholine					Phenyl		*N*-methyl piperazine			Piperazine
W	C=O				$-SO_2$	C=O		C=O			C=O
X	CH	CH	N	CH	CH	CH	CH	CH	CH	CH	CH
Y	CH	CH	CH	N	CH	CH	CH	CH	CH	N	CH
Carbon linker chain length (n)	n=2	n=3	n=2	n=2	n=2	n=2	n=3	n=2	n=3	n=2	n=2
Compound	**186**	**96**	**48**	**106**	**187**	**59**	**97**	**88**	**98**	**107**	**161**

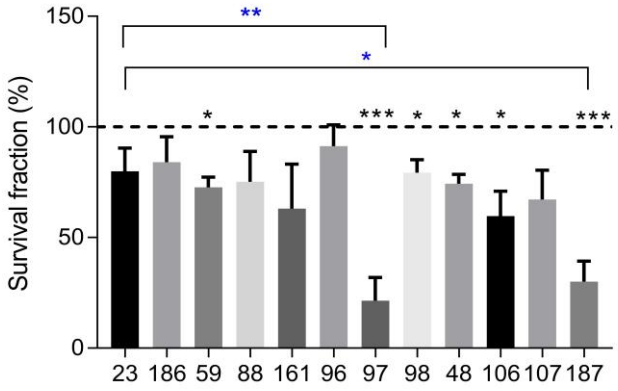


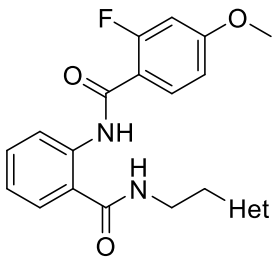
Figure 106. CFA results for compounds **23**, **186**, **59**, **88**, **161**, **96**, **97**, **98**, **48**, **106**, **107**, **187**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

Introduction of the methoxy group in compound **186** to replace the 2-F substitution on compound **23** does not improve activity. The replacement of morpholine (**186**) with phenyl ring (**59**) significantly inhibits colony formation (by a further 7.2% compared to compound **23**). Conversely, the introduction of *N*-methyl piperazine or piperazine does not enhance activity. Elongation of the carbon linker chain in compound **96** is unsuccessful, resulting in an increase of colonies formed. Introduction of more lipophilic phenyl ring and a three carbon linker chain in compound **97** results in a highly significant increase of the inhibition of formed colonies (by a further 60% compared to compound **23**). Instead, introduction of *N*-methyl piperazine and the presence of a three carbon linker chain (**98**) only improves the activity by a further 5% compared to compound **23**. Introduction of a nitrogen atom in two different positions (3 and 4) of the central ring of the molecules, such as in compounds **48**, **106** and **107** results in an improvement of the activity compared to compound **23**: the 4-nicotinamide central ring (**106**, **107**) is found to be generally more active in the inhibition of formed colonies than the 3-iso nicotinamide central ring (**48**); moreover, the simultaneous introduction of nitrogen atom in 3 position and replacement of the morpholine with *N*-methyl piperazine ring (**48**) leads to a further 25% decrease in colony formation compared to compound **23**. The introduction of the sulfonyl isoster (**187**) results in a significant inhibition of formed colony compared to compound **23** by a significant further 49.88%.

Simultaneous insertion of 2-F and 4-OCH_3 groups on the benzoyl ring was investigated to address the question if there is a synergistic effect of the two substitutions and thus an improvement in activity.

Substitutions are listed in Table 23 and results are reported in Figure 107.

Table 23. Substitutions of test compounds in Figure 107.



Heterocycle	Morpholine	Phenyl	*N*-methyl piperazine	Piperazine
Compound	**31**	**58**	**86**	**99**

As shown in Figure 107, compound **31** was found to inhibit significantly colonies formation (by a further 39% compared to compound **23**). Although statistically significant compared to negative control, compounds **86** and **99** only marginally inhibit colony formation. Replacement of morpholine with a phenyl ring (**58**) is not significant either against the vehicle control or compound **23**.

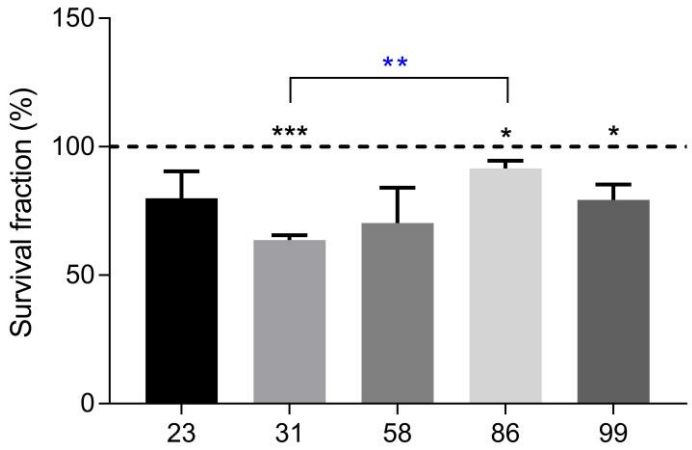
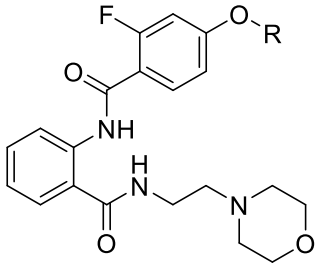


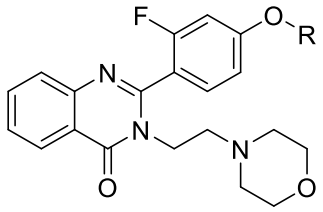
Figure 107. CFA results for compounds **23**, **31**, **58**, **86**, **99**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

Due to the interesting profile of compound **31**, a series of its analogues (listed in Table 24) were tested and results are reported in Figure 108.

Table 24. Substitutions of tested compounds in Figure 108.



i



ii

structure	i	i	i	i	ii
R	-CH_3	-H	-CH_2Ph	-CF_3	-CH_3
Compound	**31**	**127**	**123**	**105**	**222**

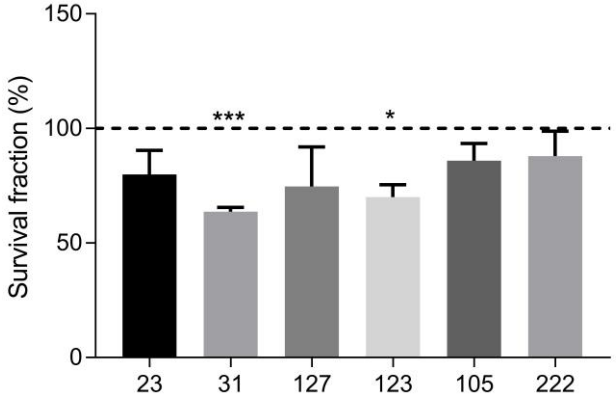
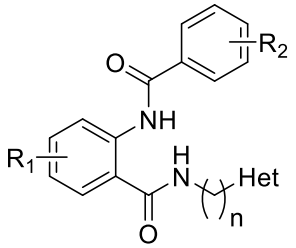


Figure 108. CFA results for compounds **23**, **31**, **127**, **123**, **105**, **222**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

Cleavage of the methyl ether (**127**) or introduction of more electronegative trifluoro-methoxy group (**105**) and closure of central ring (**222**) do not improve the activity of compound **31**. The replacement of the methyl ether with more lipophilic and sterically hindering benzyloxy group (**123**) increases the inhibition of colony formation by a further 9.87% compared to compound **23** without a statistical significant difference compared to compound **31**.

The promising results for compound **31** characterised by simultaneous introduction of 2-F and 4-OCH_3 on the benzamide ring (B), was further investigated as shown in figure 105: analogues of compound **31** with both groups in different positions of the molecule were tested in order to further improve the activity of compound **31**. Substitutions are listed in Table 25, results are presented in Figure 109.

Table 25. Substitutions of tested compounds in Figure 109.



R_1	-	-	4-OCH_3	4-OCH_3	2-F	2-F
R_2	2-F	2-F, 4-OCH_3	2-F	2-F	2-F	2-F
Heterocycle	Morpholine	Morpholine	Morpholine	Phenyl	Morpholine	Phenyl
Carbon linker chain length (n)	n=2	n=2	n=2	n=2	n=2	n=2
Compound	**23**	**31**	**34**	**64**	**35**	**65**

R_1	2-F	2-F	3-OCH_3	3-OCH_3	3-OCH_3	2-F
R_2	4-OCH_3	4-OCH_3	4-OCH_3	4-OCH_3	2-F	2-F, 4-OCH_3
Heterocycle	Morpholine	Phenyl	Morpholine	Phenyl	Morpholine	Morpholine
Carbon linker chain length (n)	n=2	n=2	n=2	n=2	n=2	n=2
Compound	**37**	**67**	**39**	**68**	**49**	**38**

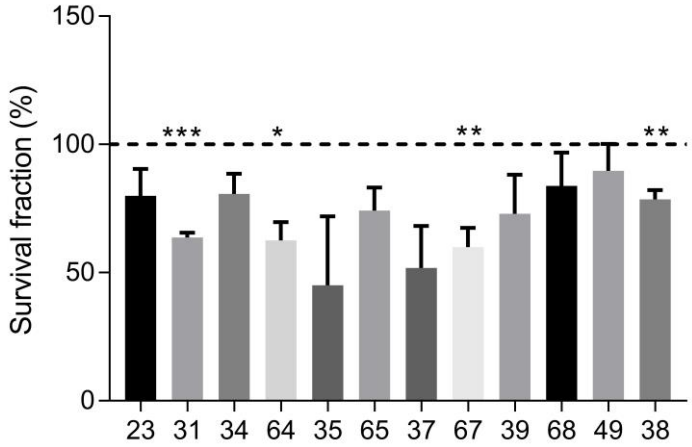
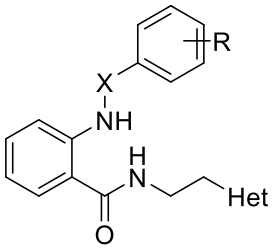


Figure 109. CFA results for compounds **23**, **31**, **64**, **35**, **65**, **37**, **67**, **39**, **68**, **49**, **38**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

As reported in Figure 109, introduction of the 4-OCH_3 group in ring B, 2-F substitution in ring A and introduction of phenyl ring (compound **64**) reduce colonies formation of 37.47% compared to the vehicle control, which was comparable to result for **31**. The same substitutions but with morpholine ring (compound **34**) leads to a decrease of activity.
Compound **67** shows a 40% reduction in formation of the formed colony compared to the vehicle DMSO and by a 20% compared to compound **23**. Compound **38** exhibits a significant reduction in colonies formation, a 21.41% decrease compared to the vehicle.

Substitution of the strong electron-withdrawing power of fluorine atom with the chlorine one was explored in *ortho*, *para* and *meta* positions and on both morpholine (**28**, **29**, **30**) and phenyl ring (**55**, **56**, **57**), as summarised in Table 26. Results are reported in Figure 110.

Table 26. Substitutions of test compounds in Figure 110.



Heterocycle	Morpholine				Phenyl		
X	C=O	C=O	C=O	$-SO_2$	C=O	C=O	C=O
R	2-Cl	3-Cl	4-Cl	2-Cl	2-Cl	3-Cl	4-Cl
Compound	**28**	**29**	**30**	**188**	**55**	**56**	**57**

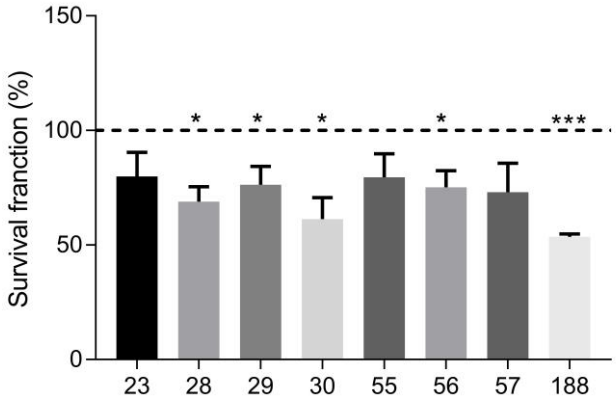
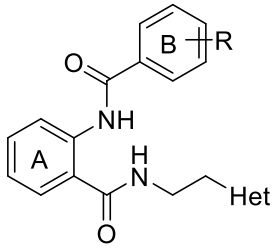


Figure 110. CFA results for compounds **23**, **28**, **29**, **30**, **55**, **56**, **57**, **188**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

As reported in Figure 110, generally, compounds with the chlorine atom in different positions and morpholine ring (**28**, **29**, **30**) present a slightly enhanced profile of activity compared to the same compounds with the phenyl ring (**55**, **56**, **57**). Compound **30** shows a 40% reduction in colonies formation compared to the vehicle. Sulfonyl isoster (**188**), with chlorine substitution in 4 position and morpholine ring, improves steadily outcome by 26.38 % reduction of formed colonies compared to compound **23** and by 46.5% compared to vehicle control.

Replacement of the fluorine atom in 2 position on ring B with highly electronegative trifluoromethylgroup in 2 and 3 position and both morpholine and *N*-methyl piperazine as heterocyles were explored. Substitutions are listed in Table 27 and results presented in Figure 111.

Table 27. Substitutions of test compounds in Figure 111.



Heterocycle	Morpholine		*N*.methyl piperazine
R	2-CF_3	3-CF_3	2-CF_3
Compound	**45**	**46**	**108**

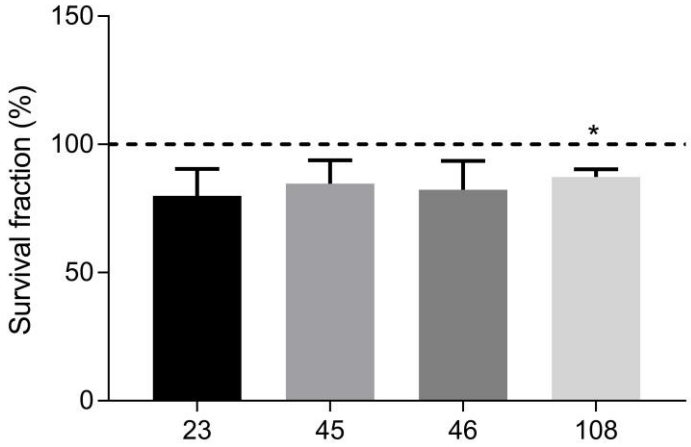
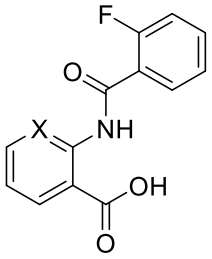


Figure 111. CFA results for compounds **23**, **45**, **46**, **108**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

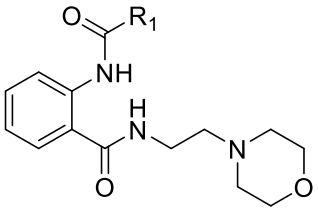
Introduction of the trifluoromethyl group to replace the 2-F group (**45**, **46**) does not improve the activity. An analogue of compound **45** but with the *N*-methyl piperazine (compound **108**) only marginally inhibits the formation of the colonies compared to vehicle DMSO.

A small series of assumed inactive compounds (**82**-**85**), from docking evaluations, were tested. A summary of the putative inactive compounds structures are listed in Table 28, while their biological results for colony forming assay are reported in Figure 112.

Table 28. Substitutions of test compounds in Figure 112.



i



ii

structure	i	i	ii	p
R	-	-	1-Naphtalyl	2-Naphtalyl
X	H	N	-	-
Compound	**82**	**83**	**84**	**85**

Their inactive profile in the colony forming assay is confirmed, as shown results reported in Figure 112.

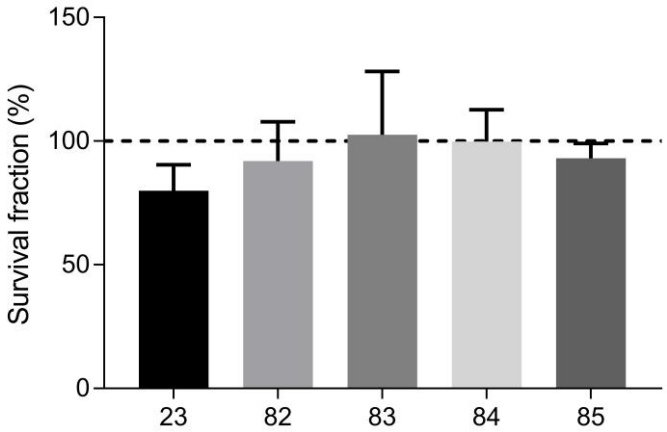


Figure 112. CFA results for compounds **23**, **82**, **83**, **84**, **85**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

A selection of compounds showing an interesting activity profile were further evaluated for their target selectivity by comparing their activity in the colony forming assay in Bcl-3 overexpressing (SW-480-WT) and non-Bcl-3 over-expressing (SW-480) cell lines, as confirmed from Western Blot in section 4.3.2.1 (Figure 74). Interestingly, the graph in Figure 113 shows that compounds **23**, **31** and **86** are inactive in the cell line without Bcl-3 and active in cell line where Bcl-3 is over-expressed, demonstrating the on-target effect of these compounds.

Compounds **82**, **83**, **84** and **85** were chosen as potentially inactive compounds to check whether they are inactive on both cell lines. Indeed, there is no inhibiting effect of these compounds on colony formation and no difference of the effect of these compounds between the two cell lines, confirming the previous results.

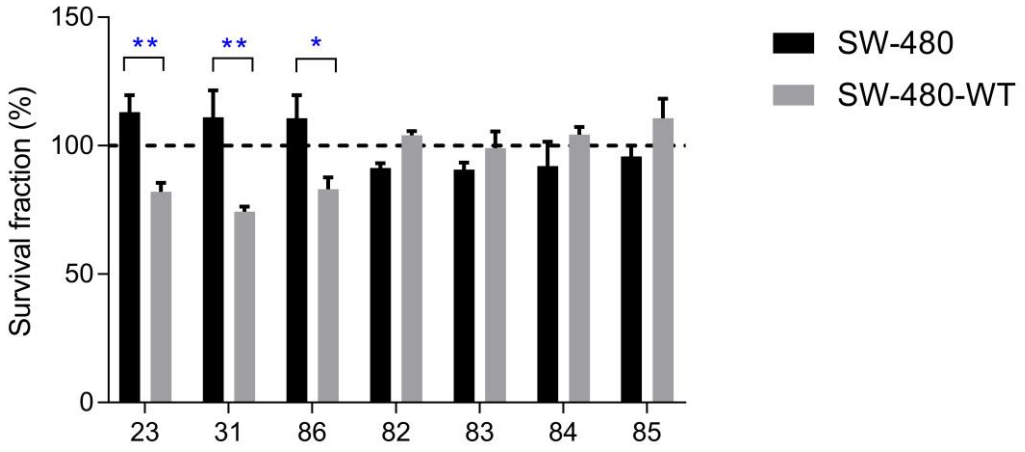


Figure 113. CFA results for compounds **23**, **31**, **82-86**. SW-480 and SW-480-WT cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

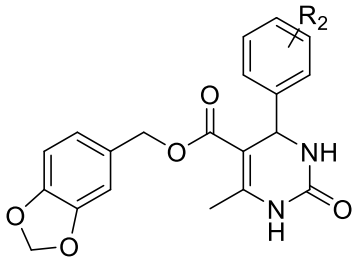
Other compounds (as for instance compound **38**, **92**, **93**, **97**, **187**, **188**) will be evaluated on the different cell lines to establish their on-target activity.

5.2.2 Colony forming assay results: analogues of compound 20

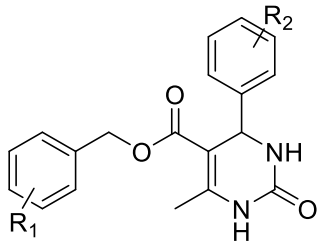
Compound **20** from the original virtual screening was used as reference compound to evaluate the activity of a series of analogues: substitutions on general structures are summarised in Table 29.

Compound **20** inhibits the formation of colonies by 20% compared to the negative control, as reported in the results in Figure 114.

Table 29. Substitutions of test compounds in Figure 114.



i



ii

structu-re	i	ii	ii	ii	ii	ii	ii	ii	i	ii
R_1	-	H	H	H	H	2, 3-OCH_3	2, 3-OCH_3	2, 3-OCH_3	-	2, 3-
R_2	3-OEt, 4-OH	4-OCH_3	4-OH	4-CF_3	3-OEt	4-CF_3	3-OEt	2-F, 4-OCH_3	2-F, 4-OCH_3	4-Cl
Compo und	**20**	**191**	**192**	**197**	**198**	**204**	**205**	**206**	**210**	**208**

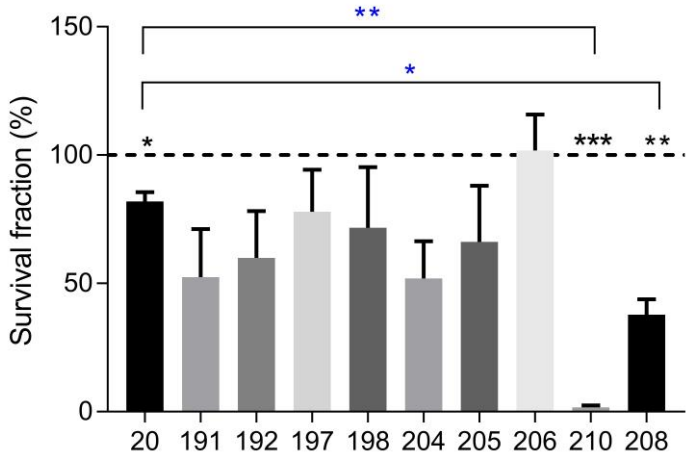


Figure 114. CFA results for compounds **20**, **191**, **192**, **197**, **198**, **204**, **205**, **206**, **210**, **208**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=4 and n=3.

Compound **210** exhibits a marked inhibition of colony formation by 98% compared to vehicle control and by 80% compared to the reference compound (**20**). Compound **208** also demonstrates a highly active profile compared to both vehicle control and reference compound. As previously demonstrated in Figure 103 and Figure 104, these two compounds do not exhibit a cytotoxic profile at 10 µM over 24 hour cell viability study in both HEK-293 and MDA-MB-231 cell lines. Due to the interesting activity profiles of compounds **210** and **208**, concentration-response studies were

performed to determine the half maximal inhibitory concentration (IC_{50}) using 4 data points of 10-fold dilution each for tested compounds (10 µM, 1 µM, 100 nM, 10 nM), as reported in Figure 115 - Figure 118. Calculations were performed on two different cell lines, both over-expressing Bcl-3, as previously shown by Western Blots in section 4.3.2.1 (Figure 74). The calculated IC_{50} for compound **210** in HCC1954 cell line is 0.56 µM, curve reported in Figure 115.

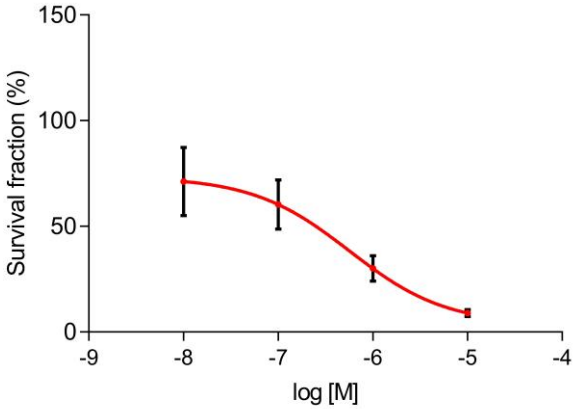


Figure 115. Concentration-response study in CFA for compound **210**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM, 1 µM, 100 nM, 10 nM (presented as log [M]). Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean ± SEM, N=3 and n=3. The IC_{50} was calculated using GraphPad Prism 7.1 by extrapolating the dose response curve, 4 parameters fitting.

The calculated IC_{50} for compound **210** in SW-480-WT is 19.22 µM and the curve is depicted in Figure 116.

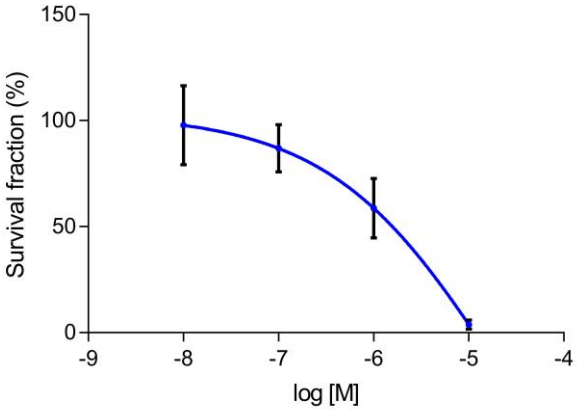


Figure 116. Concentration-response study in CFA for compound **210**. SW-480-WT cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM, 1 µM, 100 nM, 10 nM (presented as log [M]). Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean ± SEM, N=3 and n=3. The IC_{50} was calculated using GraphPad Prism 7.1 by extrapolating the dose response curve, 4 parameters fitting.

The calculated IC_{50} for compound **208** in HCC1954 cell line is 1.07 µM, curve reported in Figure 117.

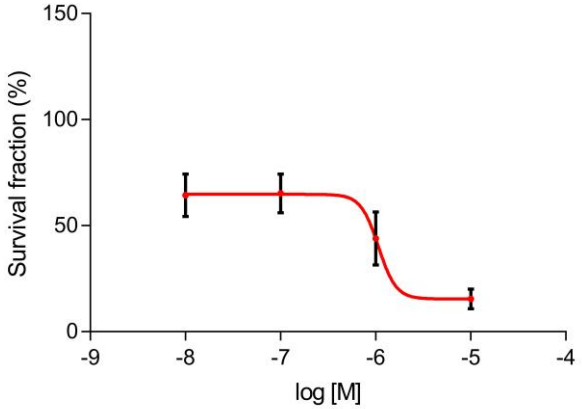


Figure 117. Concentration-response study in CFA for compound **208**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM, 1 µM, 100 nM, 10 nM (presented as log [M]). Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean ± SEM, N=3 and n=3. The IC_{50} was calculated using GraphPad Prism 7.1 by extrapolating the dose response curve, 4 parameters fitting.

The calculated IC_{50} for compound **208** in SW-480-WT cell line is 6.46 µM: although, more data points will be needed to improve the approximation of the fitting curve, these data are a good starting points in the calculation of the IC_{50} (Figure 118).

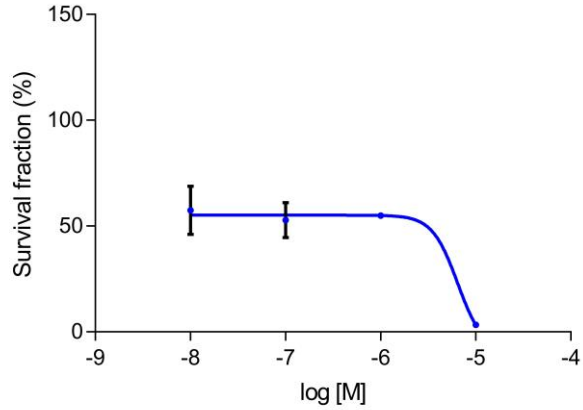
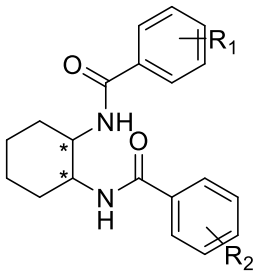


Figure 118. Concentration-response study in CFA for compound **208**. SW-480-WT cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM, 1 µM, 100 nM, 10 nM (presented as log [M]). Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean ± SEM, N=3 and n=3. The IC_{50} was calculated using GraphPad Prism 7.1 by extrapolating the dose response curve, 4 parameters fitting.

5.2.3 Colony forming results: analogues of compound 26

A few of analogues of compound **26** were tested to explore different substitutions and chirality, as listed in Table 30, results are reported in Figure 119.

Table 30. Substitutions of test compounds in Figure 119.



chirality	mixture	R,R	S,S	R, R	R, R
R_1	-COOH	2-F	2-F	3-F	2-F,4-OCH_3
R_2	-COOH	2-F	2-F	3-F	2-F,4-OCH_3
Compound	**26**	**212**	**213**	**214**	**216**

Replacement of the carboxylic acid in compound **26** with the 2-F substituent does not improve the outcomes, as shown in compound **213**, **212** and **214** (Figure 119). However, replacement of the carboxylic acid in 2 position with 2-F and introduction of a 4-OCH_3 group on the R, R enantiomer leads a highly significant reduction in colony formation compared to vehicle control and to a further 60% reduction of colony formation when compared to reference compound **26**.

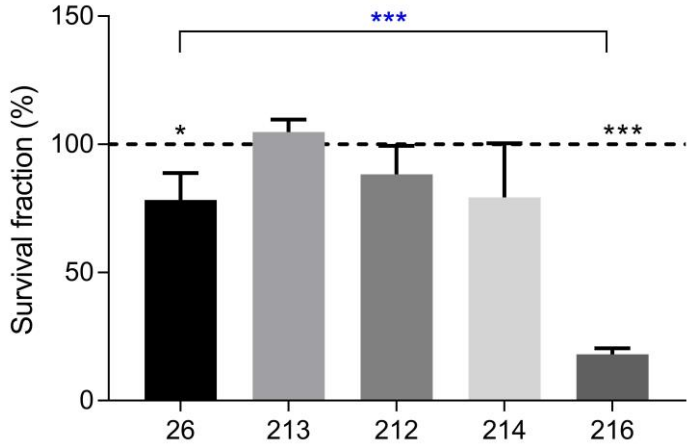


Figure 119. CFA results for compounds **26**, **212-214**, **216**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean + SEM, N=3 and n=3.

5.2.4 Colony forming assay results: analogues of compounds 26 and 27

Results of some additional analogues of compound **26** and **27** were found to be not normally distributed (Shapiro-Wilk normality test, $p < 0.05$). Results for these compounds are presented in Figure 120.

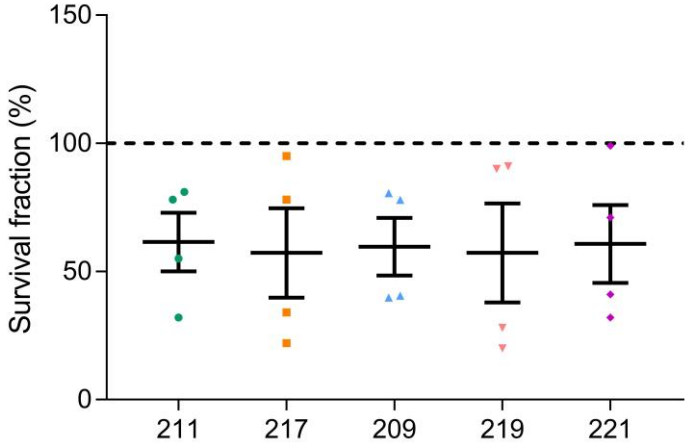


Figure 120. CFA results for compounds **211**, **217**, **209**, **219**, **221**. HCC1954 cells were plated in adherent conditions (200 cells/mL). Cells were cultured in presence of test compounds for 12 days at 10 µM. Colonies were fixed and stained with 300 µl/well of crystal violet, counted with the automated GelCount Colony Counter (Oxford Optronix Ltd) by the GelCount 1.1.8.0 software (Oxford Optronix Ltd). The number of colonies formed in presence of compounds, after 12 days, is shown as survival fraction (%). Mean ± SEM, N=3 and n=3.

For these compounds further repetitions will be necessary in the future to evaluate them biologically.

5.3 *In vitro* metabolic stability of compounds and ADME studies

All data and results shown in this section were assessed by Cyprotex LTD.

96 compounds out of 158 analogues of compound **23** have been tested for solubility (Turbidimetric Solubility Assay): lower estimated aqueous solubility is associated with compounds with a higher logP (*e.g.* compounds characterised by a phenyl ring replacing the morpholine ring in compound **23**).

Compounds **31**, **38**, **41**, **86** and **93** were then tested to assess different pharmacokinetics parameters (*in vitro* toxicology and ADME determination) in human species, including microsomal stability, protein binding and cardio toxicity. Results are summarised in Table 31.

Table 31. Results of ADME study for 5 analogues of the first scaffold, compounds: **31**, **41**, **58**, **86**, **183**.

	Metabolic stability			**Protein binding**	**hERG channel inhibition**	
Compound	*Cl int (µL/min/mg protein)*	*SE Cl int*	*$t_{1/2}$ (min)*	*Mean % recovery*	*IC_{50} (µM)*	*SE IC_{50} (µM)*
31	10.9	1.74	128	87.4	>25	-
41	49.3	1.93	28.1	-	4.48	1.35
58	17.9	3.97	77.5	90.8	>25	-
86	2.6	4	110	-	>25	-
183	2.54	4.56	545	94.5	11.7	3.39

To evaluate the stability of test compounds in phase I metabolism (metabolic stability), they were incubated with pooled human liver microsomes, and remaining compound concentration was measured at five time points over 45 minutes. Compounds **31**, **58** and **183** are moderately stable with clearance in the range of 10.9-17.9 µL/min/mg protein. Compound **41** is characterised by a higher clearance (49.3 µL/min/mg protein) which is disadvantageous as it indicates that the compound could be rapidly cleared from the body as well: as a result, the half-life time is only 28 minutes. Conversely, compound **86** exhibits a too low clearance (2.54 µL/min/mg protein) which correlates with a higher half-life time (545 minutes).

Protein binding has been determined incubating test compounds in physiological buffer and 100% species-specific plasma, using an equilibrium dialysis with two different compartments separated by semi-permeable membrane. Compounds **41** and **86** were not detectable. Compounds **31** and **58** show a favourable profile (mean % recovery ≤ 90%): interestingly compound **31** exhibits the lowest plasma protein binding than the other tested compounds, suggesting that it has the higher unbound fraction of compound available to reach the active site and then to be metabolised. If the percentage of recovery is higher than 90%, issues due to the decreased percentage of unbound compound available for therapeutic action are important to consider also in a dose determination study. For instance, compound **86** with 94.5% of plasma protein binding will be not considered in future clinical candidate studies due to its high value for the plasma protein binding which is associated to the longest half time.

Lastly, compounds have been evaluating for their cardiotoxic profile by the hERG channel inhibition assay. Briefly, mammalian cells expressing the hERG potassium channel are seeded into 384-well planar arrays and hERG tail-currents measured by whole-cell voltage-clamping. Compounds **31**, **58** and **183** exhibits an IC_{50} higher than 25 µM, indicating that they are weak hERG

channel inhibitors and this correlates with non-cardiotoxic effects in the *in vitro* model, so these compounds are likely to be non-cardiotoxic also *in vivo*.

Due to the promising results for compound **31**, this compound was further evaluated in *in vivo* studies, as discussed in section 5.4.

5.4 *In vivo* studies of compound 31

All data and results shown in this section were acquired with permission of Dr. Richard Clarkson, European Cancer Stem Cell Research Institute, Cardiff and WuXi AppTech, bio-pharma industry, China. Compound **31** was further evaluated in different *in vivo* mouse models and in different cancers. Main results are reported in Table 32.

Table 32. *In vivo* evaluation of compound **31**. TNBC =triple negative breast cancer, HER2-BC= Her2 breast cancer, CRC=colorectal cancer, NPC= Nasopharyngeal cancer.

Tumour type	reduction in growth	tumour free survival	reduction in metastasis	metastasis free survival
TNBC	46-82%	5-33%	68-93%(1)	40-60%
Her-2-BC	89%	24%	n/s	n/s
CRC	53-88%	29%	n/s	n/s
NPC	30%	n/a	n/d	n/d

Efficacy of compound **31** was proved in a mouse model of triple negative breast cancer (TNBC), where a reduction of 46-82% of the primary tumour was observed as well as a 68-93% reduction of metastasis.

Disease free survival results are reported in Figure 121, for the two doses.

Dose escalation studies to determine maximum tolerated dose in animal models have been performed by WuXi PharmaTech, China (data not reported).

Effect of compound **31** was evaluated in a mouse model (model one) of intravenously injected breast cancer cell line at two different concentrations: 3.5 mg/kg and 20 mg/kg, results are reported in Figure 121.

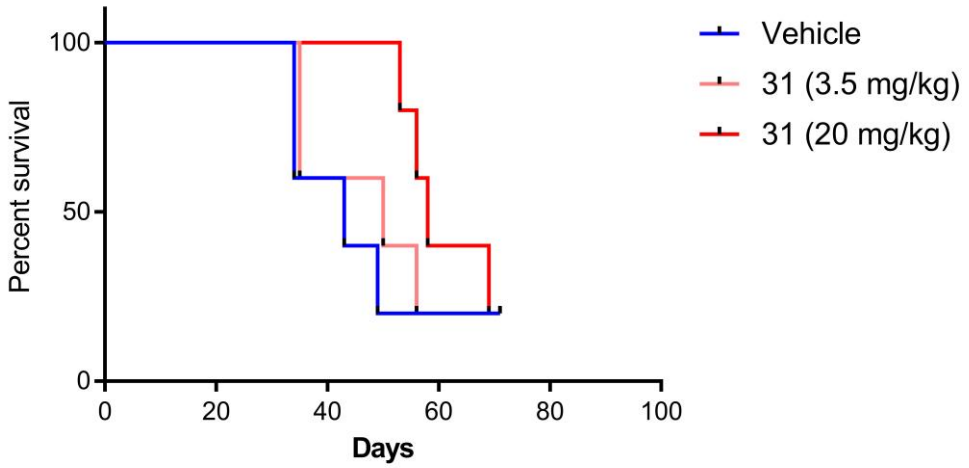


Figure 121. Effects of compound **31**, administrated orally, on metastatic spread in athymic mice injected intravenously with MDA-MB-231LUC breast cell line (3x105 cells/mouse). The experiment was performed to detect changes in metastatic spread in mice treated orally with compound one in doses 3.5 mg/kg (n = 5), and 20 mg/kg (n = 5) compared to vehicle group – 5% DMSO (n = 5). Mice were treated with tested compound or DMSO for 11 days (2 days pre-treatment + 9 days of treatment) and imaged every 2-8 days since day 12 to 28. Mice were sacrificed at day 50. Metastatic spread in time. The data are presented as the means. The data were analysed with One-way ANOVA test. (Gehan-Breslow-Wilcoxon test, P = 0.08).

Bibliography

1. Soukupova J.; Clarkson R. W. E.; Brancale A.; Westwell A. Inhibition of Bcl-3 as a novel therapeutic approach for metastatic breast cancer, 2013, Ph.D. thesis, School of Pharmacy, Cardiff University.

Section 6:

CONCLUSIONS AND FUTURE PERSPECTIVES

6. General conclusions and future perspectives

A number of analogues of the four most active compounds from previously reported high throughput virtual screening were efficiently synthesised, according to a variety of synthetic routes. The synthetic route for compound **226** will be optimised: different strategies will be tried and the final step in scheme[(28)] will be tried using different Suzuki coupling procedure, catalyst and solvent systems on the free acid after hydrolysis of compound **319**. The cytotoxic profile of the newly synthesised compounds was assessed, after successful optimisation of the general methodology for the cell viability assay. A number of compounds were tested in the colony forming assay, as predictive screening assay. Immunoprecipitation and co-immunoprecipitation experiments were designed and performed and they will be repeated to assess the on-target effect of the most promising compounds. Pharmacokinetic properties of some analogues of compound **23** (compound **31**, **41**, **58**, **86**, **183**) were investigated. Compound **31** was identified as a first clinical candidate, after its biological evaluation in several *in vitro* and *in vivo* studies.

6.1 Analogues of compound 23

Analogues of compounds **23** were synthesised introducing different electron withdrawing and electron donating group in different positions of the ring, as well as replacing the morpholine ring with different heterocycle and varying also the carbon linker chain length. A two and three carbon linker chain were explored, as the one carbon linker chain did not result in being interesting in docking studies (data not reported). All these systematic changes had the final aim to investigate a variety of properties such as logP, solubility, electronic effects of the different substituents on the interaction between the novel analogues and the binding pocket and, finally, their metabolism in order to individuate key features for the biological activity in this scaffold.

Different analogues of compound **23** were evaluated in the cell titer blue assay on both tumorigenic (MDA-MB-231) and non-tumorigenic (HEK-293) cell lines: none of them demonstrated to be cytotoxic at two different concentrations (10 μM, 1 μM) over 24 hours incubation time.

A number of compounds were tested also in the colony forming assay. Replacement of the morpholine ring in compound **23** with phenyl ring and *N*-methyl piperazine did not further reduce the percentage of survival colonies. Only replacement of the morpholine with the piperazine ring (compound **160**) significantly increased the activity of this analogue of compound **23**. In order to investigate whether the nitrogen of the benzamide was important for the activity of compound **23**,

compound **110** was synthesised: low yield achieved with the activated ester strategy were probably due to steric hindrance and intramolecular H bonds formation, which makes the intermediate less reactive for the last step of the amide formation. The methylated compound will be tested in the colony forming assay to assess its biological activity.

A small series of closer analogues of compound **186** was investigated: elongation of the carbon linker chain and the presence of the morpholine, N-methyl piperazine or piperazine did not enhance the activity of new analogues. Compound **187**, characterised by the presence of the sulfonyl group, exhibited a significant decrease in the percentage of the survival colonies. Interestingly, elongation of the carbon linker chain and the replacement of the morpholine with the phenyl ring (compound **97**) dramatically enhanced the activity of compound **97** in comparison with compound **186**. Interestingly, a correlation between the most active compounds and their lipophilicity may be found. Indeed, also compound **123** (characterised by the presence of the benzyloxy moiety in 4 position of the benzamide ring, and hence a logP of 3.65, twice the logP of compound **31**) exhibited a dramatic enhance of the biological activity in the colony forming assay. Again, also compound **64** and **67** (roughly 2.5 times more lipophilic than reference compound **23**) shown a significant decrease in the formation of the colonies compared to reference compound **23**. Unfortunately, this correlation may be due to a drawback of the general assay. Hence, these compounds will be further evaluated in other biological assays, such as immunoprecipitation and migration assay.

Simultaneous insertion on the 2-F atom and the 4-OCH_3 moiety were investigated to demonstrate a synergistic effects of this two substitutions on the activity of the compounds: also in this case, different heterocycle were introduced but only compounds **31** (characterised by the 2 carbon linker chain and the morpholine ring**)** proved to be the most active. Interestingly, compound **127,** which it could be a putative metabolite of compound **31**, did not result to be more active than compound **31**. Simultaneous introduction of the fluorine atom and methoxy group in different positions of the aromatic ring of general analogues were investigated: none of them results to be more active compared to compound **31** proving that only the simultaneous insertion of the fluorine atom and methoxy group in the specific position in compound **31** are able to conjugate the proper electronic and lipophilic effects necessary for the activity.

In the last attempt, introduction of the isoster -SO_2 moiety and replacement the fluorine atom with the chlorine one as in compounds **188** improved the outcomes in the colony forming assay.

The on-target effect of the compounds was successfully demonstrated by evaluation of the activity of few active analogues on two cell lines, over-expressing and not over-expressing Bcl-3 cell line (section 5, Figure 113).

Hence, further investigation in other biological assays (migration, invasion and immunoprecipitation assay) will be necessary to assess the definitive activity of the most interesting compounds, such as compounds **31**, **64**, **67**, **92**, **93**, **97**, **160**, **188**.

Few analogues of compounds **23** were evaluated in the ADME studies. Compound **31** resulted to exhibit the best properties, balancing a promising activity with good ADME properties. Hence, it represents a first clinical candidate.

Moreover, three different salts version of compound **31** were prepared to increase its solubility and they will be confirmed by elemental analysis.

Compound **227** – **229** were successfully synthesised using a combination of the activated ester strategy and they will be tested using the Nanotheters technology.

6.2 Analogues of compound 20

A series of analogues of compounds **20** were synthesised using a general procedure for the one pot Biginelli's reaction and different benzyl ketoesters were investigated initially. The synthesis of the β-keto ester was successfully achieved, with the conventional heating in solvent and catalyst free conditions. In order to shorten the reaction time, both reaction steps will be tried with different catalyst system and the microwave irradiation.

First, the benzo[d][1,3]dioxol-5-ylmethanol was replaced with both the benzylic alcohol and the (3,4-dimethoxyphenyl)methanol group, which represents the closest metabolite of the benzo[d][1,3]dioxol group. Different electron-withdrawing and electron-donating groups were introduced in the ring originating from the benzaldehyde in order to have a panel of different lipophilicity, solubility and electronic effects on the final compounds to assess which features were important for the activity.

None of the compounds results to be cytotoxic in both tumorigenic (MDA-MB-231) and non-tumorigenic (HEK-293) cell line.

All the compounds were tested as racemic mixtures: future investigations will include the synthesis of the enantiomeric pure compounds and their biological evaluation in the different biological assays, such as the cell titer blue assay and the colony forming assay.

Among analogues of this second scaffold (compound **191** – **210**), compound **208** and **210** shown an interesting profile of activity on the colony forming assay and for this reason their IC_{50} was evaluated. They will be further investigated in *in vitro* and *in vivo* studies, to confirm their biological activity (such as the immunoprecipitation to assess the on-target activity and migration assay). Moreover,

they will be tested on the cell titer blue assay over a longer incubation time of the compounds with the cells (48, 72, 96, 120, 144 hours) to ensure that the biological effect is not due to any cytotoxic effects. Moreover, compound **208** and **210** will be tested on a variety of metastatic breast cancer cell line (such as the MDA-MB-231, MDA-MB-436, MCF-7) to assess in which subtype of breast cancer the compounds will result to have an interesting activity. Further substitutions and chemical modifications will be established and new analogues will be synthesised to define structure-activity relationships. Moreover, the ester will be replaced by amide or a 2 carbon linker chain to avoid stability problems due to the cleavage by esterases *in vivo*.

6.3 Analogues of compound 26

Few analogues of compounds **26** were successfully prepared and few of them tested: none results to be cytotoxic in the cell titer blue assay. Surprisingly, the simultaneous presence of the 2F, 4-OCH_3 substitutions on the benzamide ring in compounds **216** resulted in the lowest percentage of colony formed. Moreover, being this one the R, R- enantiomer, it will be necessary also to test the S, S one to assess whether the stereochemistry of the chiral centers will strongly affect the biological activity of tested compounds.
These compounds will be further evaluated in both cell titer blue assay and colony forming assay. The on-target activity will be proven by immunoprecipitation experiments.
Moreover, compounds **211** and **217** will be tested few more times in the colony forming assays in order to have a normal distribution of results and being able to assess the real activity of these analogues of compound **26**.

6.4 Analogues of compound 27

Analogues of compound **27** were successfully synthesised in a 4 steps synthetic route. Further modification will be explored also on the benzene ring of the 3-chlorobenzo[d]isothiazole 1,1-dioxide. Few analogues of compound **27,** not cytotoxic in the cell titer blue assay, will be tested again in the colony forming assay to assess their biological activity on this first predictive screening assay.

Section 7

MATERIALS AND METHODS

7.1 Cell line

Different cell lines were used: MDA-MB-231, HEK-293, HCC1954, SW-480. MDA-MB-231 is the widely used cell line for *in vitro* studies of hormone dependent breast cancer as a model of cancer metastasis. It is a triple-negative (ER-, PR-, no HER2 over-expression) cell line, strongly over-expressing EGFR. MDA-MB-231 cells form highly malignant, invasive cancers *in vivo.* The MDA-MB-231-WT was generated by transfection of parental cell line to over-express Bcl-3. The MDA-MB-231-ANK mutant over-expresses Bcl-3 and it lost the ability to bind to p50 due to three mutations on the Bcl-3 sequence: lysine residues 167, 170, 173 were mutated with alanine residues. The sequence of Bcl-3 MDA-MB-231-WT and selected binding mutant MDA-MB-231-ANK were confirmed by sequencing as reported previously.[1]

HEK-293 is a Human Embryonic Kidney 293 cell line, deriving from embryonic kidney cells grown in tissue culture and from still born animals. Because HEK-293 may be grown and transfected very readily, they are widely used in a biological laboratory. HEK-293 have an undetectable basal level of Bcl-3 protein. Over-expressing Bcl-3 HEK-WT-7 clone was generated and confirmed by sequencing, as previously reported.[1]

HCC1954 is a triple negative metastatic IIA, GRADE 3, ductal carcinoma of a 61 years old female, characterised by higher endogenous expression level of Bcl-3.

All of these three cell lines were transfected in Dr. Richard Clarkson research group (Hadyn Ellis Building, European Cancer Stem Cells Research Institute).

SW-480 is a Dukes' type B, colorectal adenocarcinoma of a 50 years old male, transfected to generate Bcl-3 over-expressing cell line (SW-480-WT), kind gift by Professor Ann Williams (School of Cellular and Molecular Medicine, University of Bristol).

Bcl-3 protein in all the wild type clones of different cell lines is tagged with a FLAG peptide at *N*-terminus, as previously reported.[1] Western Blots were performed to check Bcl-3 and p50 expression level in each cell lines, every 2 months.

7.2 Maintenance of cultured cell

Human breast cancer cells MDA-MB-231 and HCC1954 were cultured in complete growth media: RPMI-1600 1X (Gibco®) media was supplemented with 10% v/v Fetal Bovine Serum (FBS, Sigma Dorset UK) and L- Glutamine (2mM, Invitrogen). MDA-MB-231-WT and ANK mutant were

maintained in complete growth media RPMI-1600 1X, enriched with 300 µg/mL of Geneticin® Selective Antibiotic (G418 Sulfate, Gibco, Thermo Fisher scientific). HEK cells and sub-lines were cultured in Dulbecco's Modified Eagle Medium (Gibco®) enriched with 10% v/v Fetal Bovine Serum (FBS, Sigma Dorset UK) and L- Glutamine (2mM, Invitrogen). The HEK-WT-7 was maintained in selective media made up of the Dulbecco's Modified Eagle Medium (Gibco®), enriched with 0.3% Geneticin® Selective Antibiotic (G418 Sulfate, Gibco, Thermo Fisher scientific). SW-480 cell line was maintained in Dulbecco's Modified Eagle Medium (Gibco®) enriched with 10% v/v Fetal Bovine Serum (FBS, Sigma Dorset UK) and L- Glutamine (2mM, Invitrogen). SW-480-WT was maintained in selective media made up of Dulbecco's Modified Eagle Medium (Gibco®), enriched with 500 µg/µL and 1000 µg/µL of Geneticin® Selective Antibiotic (G418 Sulfate, Gibco, Thermo Fisher scientific). The composition of complete growth media allowed survival, growth and division of cells. Cells were incubated in a T25, T80 or T160 culture tissue flask (Nunc, Leics, UK) at 5% CO_2 atmosphere at 37 °C and split when they became 80-90 % confluent, to keep a suitable plating density. Growth media was removed by aspiration and 0.25% Trypsin/EDTA (Invitrogen) was added (1 mL for T25 flask, 3 mL for T80 flask, 5 mL for T160 flask); cells were incubated at 37 °C and 5% CO_2 for 5 - 10 minutes. Cells were detached and 5 ml for T25 tissue culture flask of growth media was added to the cells. These were then split at an appropriate ratio by removal of cell suspension excess into waste. The remaining volume of cell suspension was made up to 5 mL for T25 (15 mL for T80, 30 mL for T160 tissue culture flasks) with complete growth media.

7.3 Long-term cell storage

In order to have sufficient aliquots of low passage number cell lines, cells were cryo-stored. Cells were detached as described in section 8.2 using 0.25 % Trypsin (Invitrogen), re-suspended in complete growth media in 15 mL falcon tube, centrifµged at 1200 rpm for 5 minutes at 20 °C. The supernatant was removed, cells re-suspended in the freezing media (complete growth media enriched with 10% v/v dimethyl sulfoxide –Sigma, Dorset, UK) and aliquoted into 1 mL cryo tube vials (Nunc, Leics, UK). The cryo tube vials were placed into a container with iso-propanol at room temperature in order to achieve a slow freezing at -80 °C. After 24 hours, the cryo tube vials were transferred into liquid nitrogen storage. When cells were retrieved from cryo-storage, they were quickly defrosted to 37 °C in a water bath, re-suspended in 10 mL of complete growth media, centrifµged at 1200 rpm at 25°C for 5 minutes. The supernatant was removed and the pellet was re-suspended in 5 mL of complete growth media and transferred to a T25 tissue culture flask.

7.4 *Mycoplasma* Detection assay

The widely method used is the *Mycoplasma* detection assay, a PCR based test: the PCR Mycoplasma Test Kit 1/C (Promokine). An agarose gel electrophoresis is performed.
The general steps of this assay are:

1) centrifµge the culture cell at the minimum rate (2x 1000) in order to separate the dead cells by the alive ones;
2) centrifµge the supernatant at maximum spin (13 x 1000) in order to deposit the eventual *Mycoplasma* DNA on the bottom of the test tubes;
3) preparation of the enzyme (Taq Polymerase): the enzyme is in a buffer solution and it is used for the amplification of the DNA of the *Mycoplasma*, if it is present in the culture cell;
4) preparation of a gel made of triacetate ETDA buffer, agarose and a fixative for nucleic acid;
5) gel run for 30 minutes at 180 V;
6) DNA bands are visualised thanks to a GelDoc (BioRad) and size of the bands is identified by comparison with the DNA molecular weight (the weight of DNA of the *Mycoplasma* is 504-519 bp, approximately 150 kDa).
7) Absence of band in correspondence of the one of the Mycoplasma sample means that the culture is not contaminated.

7.5 Plasmocin treatment: against Mycobacterium infection

In order to cure *Mycoplasma*-positive cell lines, Plasmocin Treatment was used. Plasmocin is a recently, new anti-mycoplasma antibiotic. It is made up of two bactericidal components: a quinolone, which is bactericidal inhibiting nucleic acid synthesis; a macrolide, a bacteriostatic, inhibiting the protein synthesis, which acts on the protein synthesis machinery by interfering with ribosome translation. The treatment was carried out for 3 weeks at a concentration of 25 µg/mL. The cells were splitting into medium containing 25 µg/mL of Plasmocin Treatment every three days. After three weeks of treatment, the cell lines were tested in order to detect Mycoplasma using a PCR Mycoplasma Test Kit 1/C (PromoKine).

7.6 Cell counting

Cells were treated with trypsin to detach them by incubation for 5 minutes at 5% CO_2 and 37 °C; after addition of 5 mL of complete growth media, 10 µL of cell suspension was loaded on haemocytometer and cells were manually counted using a microscope (magnitude 4x). Cells with a bright circle were alive and they could be counted. The Petroff-Hausser Counting Chamber was used and the cells in

the four big squares in the four corners were counted. The number of cells was obtained as the average of the four calculations. To obtain the number of cells in 1 mL it was necessary to multiply for 5000 (factor of conversion). Automated cell counting Luna instrument: 10 µL of cell suspension are inserted into the Automatic Cell counter and calculation was performed, according to manufacture's instructions.

7.7 Cell Titre Blue viability assay

MDA-MB-231 and HEK-293 cells were cultured in the T80 flask to reach a 90% confluence, as plate density. Cells were detached using 0.25% Trypsin (Invitrogen), incubated for 5 minutes at 37 °C and 5% CO_2, complete growth media was added and the 10 mL cell suspension was moved in a falcon and centrifµged at 1200 rpm for 5 minutes. The supernatant was removed and the pellet was re-suspended in 9 mL of media. In the 96-well plate, 90 µL of cell suspension at a seeding densities of 1 x 10^6 cells per well is added. Compound samples were prepared at two different concentrations (10 µM and 1 µM) and 10 µL of each concentration is seeded in each well. Three different negative controls were used: DMSO 0.01 % and 0.001 % and the lead compound **23**. As positive control, carboplatin (30% w/v) was used. Hence, the 96-well plate was incubated for 24 hours at 37 °C and 5% CO_2. 20 µL of Cell Titre Blue reagent (Promega, Southampton, UK) was added and incubated for 2 hours at 37 °C in 5% CO_2. Fluorescence was measured by setting excitation/emission wavelengths to 560/590 nm on a Fluorostar Optima plate reader (BMG Labtech, Bucks, UK).

7.8 Protein preparation

7.8.1 Protein extraction from cells

Cells were prepared and proteins were extracted from cells. The media was aspirated from tissue culture flasks, ice cold PBS (Gibco®) was added (5 mL for T25, 10 mL for T80 and 15 mL for T160) and cells were removed with a cell scraper (Nunc, Leics, UK). The cell suspension was moved in a 15 mL falcon tube and centrifµges at 11000 rpm for 5 minutes at 25 °C. The pellet was used for protein extraction immediately or stored at -20 °C prior use.

7.8.1.1 Whole cell protein extraction (denaturing condition)

To run a Western Blot, cell extracts were isolated using RIPA buffer with the addition of complete mini protease inhibitor protease tablets (Roche, UK), 10 mM sodium fluoride (Fluk Bochmika), 1 mM sodium pyrophosphate and 1 mM sodium orthovanadate (Sigma, UK). Cell pellets from section 7.8.1 were resuspended in appropriate volume of RIPA buffer (200 µL). Cell suspension was passed

throμgh a 23G needle 10 times to ensure complete cell lysis, incubated on ice for 30 minutes, centrifμged at 10000 rpm for 15 minutes at 4 °C to pellet cell debris and the supernatant was aliquoted into fresh tubes and use immediately or stored at -20 °C or used immediately for protein concentration determination.

Composition of RIPA buffer: 50 mM Tris pH 8 (Sigma), 150 mM sodium chloride (Sigma), 1% v/v sodium dodecyl sulphate (Sigma), 0.5% w/v sodium deoxycholate (Sigma).

7.8.1.2 Whole cell protein extraction (non-denaturing condition)

Cell extracts were suspended in a non denaturating lysis buffer to investigate the protein-protein interaction. Complete mini protease inhibitor tablets (Roche, UK), 10 mM sodium fluoride (Fluke Bochmika), 1 mM sodium pyrophosphate and 1 mM sodium orthovanadate (Sigma, UK). Cell pellets from section 8.8.1 were resuspended in appropriate volume of non denaturating buffer (200 μL). The cell suspension was transferred to a micro centrifμge tubes, sonicated on ice (3 times, 5 seconds) and centrifμged at 10000 rpm for 10 minutes at 4 °C to pellet cell debris and the supernatant was aliquoted into fresh tubes and use immediately or stored at -20 °C or used immediately for protein concentration determination.

Composition of non denaturating buffer: 20 mM Tris pH 7.5 (Sigma), 150 mM sodium chloride (Sigma), 1% v/v Nonidet-P40 (Roche), 1 mM EDTA pH 8 (Fisher Scientific), 1 mM EGTA pH 8.0 (Fluka Biochemika).

7.9 Determination of protein concentrations - bicinchoninic acid assay (BCA assay)

BCA assay kit (Pierce, Loμghboroμgh) was used to determine protein concentration. 12.5 μL of protein samples were added to 100 μL of BCA reagent and cultivated at 37 °C for 30 minutes. A blank sample was prepared by addition of 12.5 μL of RIPA buffer into 100 μL of BCA reagent. Colorimetric change was measured using a nanodrop spectrophotometer (ND-100; Labtech International) at 562 nm. A standard curve was determined from a set of known concentrations of bovine serum albumin (0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 5 mg/ml) and used to extrapolate the relative concentration of protein extracts.

7.10 Western Blot

Cells were routinely checked for Bcl3 expressions by Western Blots.

7.10.1 Preparation of protein samples for Western Blot

Protein extracts were diluted with RIPA buffer to a final volume of 24 µl. Hence, 6 µl of 5x Laemmli buffer was added to each sample and proteins were denaturated by heating to 95°C for 5 minutes.

7.10.2 Casting of polyacrylamide gels

SDS-PAGE consisted of resolving and stacking phases (composition reported in Figure), while the % of acrylamide used in the resolving phase depended on the size of proteins to be analysed. Proteins used in this thesis were about 50 kDa, than the 10% resolving gel was used. SDS-PAGE gels were set using Mini Protein III (Bio-Rad) gel casting apparatus. Casting plates were cleaned with 70% ethanol before being assembled in a gel casting apparatus and filled to approximately two thirds with the resolving gel. Once set, the stacking gel was poured into the resolving gel and 10 well comb was inserted immediately. The combs were removed and the wells were rinsed with distilled water.

10% Resolving gel	**4% Stacking gel**
4.1 ml distilled water MilliPore	6.1 ml distilled water MilliPore
3.3 ml 30% Acrylamide (Sigma)	1.3 ml 30% Acrylamide (Sigma)
100 µl 10% w/v ammonium persulphate (Sigma)	100 µl 10% w/v ammonium persulphate (Sigma)
2.5 ml 1.5 M Trs-HCl pH 8.8 (Sigma)	2.5 ml 0.5 M Trs-HCl pH 6.8 (Sigma)
100 µl 10% w/v sodium dodecyl sulphate (Sigma)	100 µl 10% w/v sodium dodecyl sulphate (Sigma)
15 µl TEMED (Sigma)	15 µl TEMED (Sigma)

Figure 122. Composition of resolving and stacking gel.

7.10.3 Gel electrophoresis

Protein separation was performed using the Mini-Protean III (Bio-Rad) electrophoresis tank containing Tris-Glycine running buffer. Protein molecular weight marker or ladder (PageRuler Plus) was loaded into the first lane of each gel and prepared protein samples were loaded into appropriate remaining wells. Gels run for 1 hour at 180V.

7.10.4 Transfer of proteins to PVDF membranes

Before transfer, Immobilon-P polyvinylidene difluoride membrane (PVDV, Millipore) was cut to a required size and soaked in methanol for 10 seconds and washed in PBS/T (phosphate buffer saline

supplemented with 1% v/v Tween). The membrane sandwich was assembled according to manufacturer's instructions in a wet electroblotting system using sponges, filter papers, membrane and the prepared gel. Air bubles were carefully rolled out after the addition of each layer. The block was inserted into the transfer apparatus and proteins were transferred in 1x Tris-Glycine transfer buffer at 80V for 45 minutes. Buffer compositions are reported in Figure 123.

Laemmi buffer	**10x Electrophoresis buffer pH 8.3**	**1x Tris-Glycine running buffer**	**1x Tris-Glycine transfer buffer**	**Stripping buffer**
0.125 M Tris-HCl pH 6.8 (Sigma)	30.3 Trisma base (Sigma)	50 mL 10 electrophoresis buffer	100 ml 10x electrophoresis buffer	62.5 mM Tris-HCl pH 6.8 (Sigma)
4% w/v SDS (Sigma)	144.4 g Glycine (Sigma)	5 ml 10% w/v SDS (Sigma)	200 ml methanol (Fisher)	2% w/v SDS (Sigma)
40% v/v glycerol (Sigma)	Distilled water up to 1l	380 ml distilled water	700 ml distilled water	0.7% v/v beta mercaptoethanol (Sigma)
0.1% w/v bromophnol blue (Sigma)				
6% v/v beta-mercaptoethanol (Sigma)				

Figure 123. Composition of different buffers for Western Blot.

7.10.5 Probing of membranes

After transfer, membranes were washed in PBS/T and blocked in milk blocking solution (5% w/v non-fat milk in PBS/T) under agitation for at least an hour at room temperature. Membranes were then incubated in 5 ml of primary antibody at 4°C on a roller mixer (Stuart, Merton, UK) overnight. After cultivation, membranes were washed in PBS/T followed by cultivation with appropriate secondary antibody. Membranes were then washed again in PBS/T prior to protein detection by enhanced chemiluminescence.

Primary Antibody	Target	Species	Dilution rate	Antigen size	Supplier/Catalogue number
	Bcl-3	Rabbit	1:200	~ 50 kDa	Santa Cruz (sc-185)
	Bcl-3	Rabbit	1:500	~ 50-60 kDa	Proteintech (23959-1AP)
	p-50	Rabbit	1:200	~ 50-105 kDa	Abcam (ab7549)
	p-50	Rabbit	1:500	~ 50 kDa	Proteintech (14220-1AP)
	p-52	Rabbit	1:1000	~ 52 kDa	Cell signalling (4882s)
	p-52	Rabbit	1:200	~ 52 kDa	Santa Cruz (sc-848)
	AntiFlag	Rabbit	1:400	~ kDa	Sigma Aldrich (F7425)
	AntiFlag	Rabbit	1:1000	~ kDa	Cell Signalling (2368s)
	GADPH	Mouse	1:1000	~ 37 kDa	Santa Cruz (32233)
Secondary Antibody	Rabbit	Goat	1:2000	-	DAKO (P0448)

Figure 124. Composition of different buffers for Western Blot.

7.10.6 Visualisation of protein bands

Immobilised immune-labelled proteins were detected using ECL or ECL Prime reagent according to manufacturer's instruction. Prepared ECL/ECL+ solutions were mixed well and cultivated for 1 minute, before addition on the membrane (1 ml per membrane). After the removal of excess reagent, the membrane was placed in a light-proof cassette, exposed to light sensitive films (Amersham Hyperfilm ECL) for 3 minutes and developed on an automatic film processor (Xograph Compact X4). Processed films were then realigned with the original membrane and the target protein was identified by comparison to the molecular weight marker.

7.10.7 Stripping and reprobing of membranes

Membranes were stripped before being cultivated with subsequent antibody f interest. Membranes were washed in PBS/T and incubated in stripping buffer for30 minutes at 55 °C under gentle agitation. Membranes were washed in PBS/T, blocked in milk blocking solution (5% w/v non fat- milk in PBS/T) and probed with a required antibody. Visualisation was performed in section 8.10.6.

7.11 Elisa on cell lysate using Pre-coated ANTI-FLAG® High Sensitivity, M2 coated 96-well plates

Non denaturating cell lysate was diluted with the appropriate buffer (TBS/T or PBS/T or PBS) to a concentration of 1 µg/µl and 100 µl was added onto ANTI-Flag coated flat bottom ELISA plates (Sigma). Samples were added in triplicates, TBS or TBS/T 0.5% or PBS 1Xor PBS/T 0.5% were used as a negative control. The plate was cultivated at 37 °C for an hour followed by 3x 200µl washes with TBS/T or PBS. Primary antibody, either Bcl-3 for Indirect ELISA or p50 for Sandwich ELISA were added in known volumes (25 µL) and concentrations to each well, cultivated covered from light for one hour at room temperature. Another 3x 200 ml washes with TBS/T or PBS were performed before incubation with Alkaline phosphatase (AP) conjµgated secondary antibody. Para-nitrophenylphosphate solution (pNPP, Santa Cruz) was prepared according to manufacturer's instructions (5 mg of disodium salt in 5 ml of pNPP substrate buffer). After cultivation with the secondary antibody, the wells were washed 3x200 µl TBS/T, pNPP was added (50 µl/well) and cultivated for an hour covered from light at room temperature. The reaction was stopped by addition of 3N NaOH (20 µl/well) and the colorimetric changes were measured at 405 nm using a Clariostar plate reader. Different antibodies used are reported in section 4.3.

7.12 Immunoprecipitation and co-Immunoprecipitation

7.12.1 Cell preparation for IP

Cell lines to use: MDA-MB-231 and MDA-MB-231-WT. IgG control (IgG catalogue number I5006, Sigma Aldrich) was a white powder: weight 2 mg and dilute it in 2 mL, store the rest at 4 °C.
The MDA-MB-231 was in RPMI-1600 1X (Gibco®) supplemented with 10% v/v Fetal Bovine Serum (FBS, Sigma Dorset UK) and L- Glutamine (2mM, Invitrogen); the MDA-MB-231-WT were in 10% v/v Fetal Bovine Serum (FBS, Sigma Dorset UK) and L- Glutamine (2mM, Invitrogen), enriched with 0,3% Geneticin® Selective Antibiotic (G418 Sulfate, Gibco, Thermo Fisher scientific). Cells were growing in 10 cm^2 dish for 48 hours. Cells suspensions were collected as described in section 7.8.1.3, counted as described in section 8.6. The protein concentration was determined as shown in section 7.9.

7.12.2 Preparation of buffers

Buffer composition is reported in Figure 125.

20 mM DMP	Wash buffer (PBS/T 0.5%)	Coupling buffer
20mM DMP solution by adding 5.18mg DMP per 1mL coupling buffer and mix. Optimal working range pH = 8.2 - 9.	PBS with 0.02% Tween-20. Add 20µL Tween-20 to 100mL PBS pH7.4.	0.2M triethanolamine in PBS with 0.01% Tween-20: add 1.52mL triethanolamine per 50mL PBS. Add 5µL Tween-20 and mix. Add concentrated HCl to pH9. Check the pH again after addition of DMP.

Quenching buffer	Elution reagent	Co-IP Lysis Buffer
50mM ethanolamine in PBS with 0.01% Tween-20. Add 155.9µL ethanolamine to 50mL PBS. Add 5µL Tween-20 and mix.	0.2M glycine pH2.5 with 0.01% Tween-20. Make up in water, add concentrated HCl to pH to 2.5.	20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM b-glycerophosphate, 1mM Na3VO4, 1µg/ml leupeptin, 10% glycerol, 0.5mM DTT (added fresh from 1M stock in water). Add protease inhibitors and 0.5mM DTT to the co-IP lysis buffer and mix

Figure 125. Different buffers for IP and co-IP.

7.12.3 Crosslinking of p50 antibody to Dynabeads

Dynabeads Protein G were provided at 30mg/mL and 1mg beads binds ~8µg IgG. Therefore, 1mg = 33µL and this should bind ~8µg antibody.

The p50 antibody (Proteintech, 14220-1-AP) was provided at 28 µg/150 µL. Dilute 60 µL of dynabeads in 77.4 µL of p50 antibody (20 µL per each sample you are going to use).

1. Vortex/mix dynabeads in their vial until completely re-suspended (60 seconds).

2. Take 60 µL dynabeads into a fresh Eppendorf. Apply to magnet, wait ~30 seconds and then discard the supernatant to discard the bead storage buffer. Remove from magnet and re-suspend Dynabeads in 60µL PBST.
3. Add a further 240 µL PBST and 77.4 µL p50 antibody to the beads, taking the final volume to 1mL.
4. Rotate at room temperature for 2 hours.
5. Following antibody/bead incubation, apply the beads to the magnet and remove the supernatant.
6. Wash beads by adding 1mL Wash buffer/PBST and vortexing for 10 seconds and inverting a few times. Apply to magnet and remove supernatant. Do this three times. Leave the beads in the final volume of wash buffer until you have prepared the DMP.
7. Wash beads three times in Coupling Buffer. Repeat step 6 but with 0.2M triethanolamine in PBS with 0.01% Tween-20.
8. Prepare the first batch of 20mM DMP solution by adding 5.18mg DMP per 1mL coupling buffer and mix. Check pH = 8.2-9.
9. Add the first 1mL DMP in coupling buffer to the beads and rotate at room temperature for 30 minutes. In the last 5 minutes, prepare another fresh batch of DMP in coupling buffer as before. Check pH = 8.2-9.
10. Remove the previous 1 mL, add the second 1mL DMP in coupling buffer to the beads and rotate at room temperature for 30 minutes.
11. To quench the reaction, add 1mL quenching buffer (50mM ethanolamine in PBS with 0.01% Tween-20) and rotate at room temperature for 30 minutes.
12. Repeat the quenching step: add 1mL quenching buffer (50mM ethanolamine in PBS with 0.01% Tween-20) and rotate at room temperature for a further 30 minutes.
13. Wash the beads three times (for 1 minute each time) by vortexing and inverting in 0.2M glycine pH2.5 (elution reagent) to remove any uncrosslinked antibody from the beads.
14. Resuspend the beads in the original volume (in this case 60µL) using TBST and store at 4°C until you are ready to use them.

7.12.4 Preparation of ANTI-FLAG BEADS (M8823-SIGMA ALDRICH)

1. Thoroughly re-suspend the resin by gentle inversion.
2. Remove 300 µL for use.
3. Equilibrate beads by re-suspending with 5 packed gel volumes of TBS (1500 µL).

4. Place tube in the appropriate magnetic separator to collect the beads. Remove and discard the storage buffer/TBS mixture.
5. Equilibrate beads by re-suspending with another packed gel volumes of TBS.
6. Mix thoroughly.
7. Place tube in the appropriate magnetic separator to collect beads.
8. Remove and discard TBS buffer. Repeat steps 3 and 4 once. Allow a small amount of buffer to remain on the top of the beads.
9. Divide the resin according to the number of samples.
10. Run the co-IP.

7.12.5 co-IP protocol

Lysis

1. Wash cells twice in ice-cold PBS
2. Add Co-IP Lysis Buffer, ensure all cells are covered, scrape on ice (250µl for a T25)
3. Retrieve lysate into an Eppendorf, keep on ice for around 5-10 minutes vortexing occasionally.
4. Sonicate using 4x 30 second pulses in the cold to ensure complete lysis.
5. Centrifuge nuclear lysate for 10 minutes at high speed (15000 rpm) at 4°C and retrieve the supernatant to a fresh Eppendorf.
6. BCA protein concentration determination as described in section 8.8.2.
7. On ice, wash and prepare the beads by removing the desired amount from the stock (e.g. 33µL per each single IP), apply to the magnet, discard the supernatant. Resuspend the beads in 500µL co-IP buffer, vortex and then reapply to the magnet. Remove the supernatant and resuspend the beads in co-IP buffer to the original volume (33µL).
8. Co-IP step. Add antibody bound beads to the protein lysates (33µL beads in 1000µg protein) and rotate overnight at 4°C overnight.

Elution phase

9. To wash the co-IPs, apply them to the magnet (on ice). Save the first supernatant – this is the immunodepleted lysate. Wash the beads by gently adding 500µL co-IP buffer and mix by rotating at 4°C for 1 – 5 minutes. Repeat this for a total of 6 washes.
10. After the final wash, remove as much supernatant as possible. To elute the pulled-down complexes, boil/heat at 95°C beads in 2x laemmli buffer for 5 minutes. Store at -20°C until ready to run the western blot.

Western Blot

As described in section 7.8.3. Primary antibody used are reported in Figure 126.

Target protein	*Antibody*		
	Species	*Dilution*	*Catalogue number*
Bcl-3	Rabbit	1:500	Proteintech (23959-1AP)
p-50	Rabbit	1:500	Proteintech (14220-1AP)

Figure 126. Different antibodies for Western Blot

Stripping the membranes

Prepare the stripping solution: 2 mL SDS 10X, 1mL 0.625 M TRIS pH 6.8, 7 mL water. Soak the membrane at 42 °C for 30 minutes. Wash 6 times, 5 minutes each with TBST 0.5%.

Blocking

Repeat the blocking step for the second Western blot. 120 minutes incubation with 5% milk in TBST 0.5% v/v at room temperature.

Western Blot

As described in section 7.8.3.

Target protein	*Antibody*		
	Species	*Dilution*	*Catalogue number*
Bcl-3	Rabbit	1:500	Proteintech (23959-1AP)
p-50	Rabbit	1:500	Proteintech (14220-1AP)

Figure 127. Different antibodies for Western Blot.

7.13 Colony forming assay

HCC1954 cells were maintained as monolayers in T25 tissue culture flasks in media as described in section 8.2. Single cell suspensions are prepared by adding 0.25% Trypsin/EDTA (Invitrogen) (1 mL

for T25 flask, 3 mL for T80 flask, 6 mL for T160 flask) in the tissue culture flasks; the cells were incubated at 37 °C and 5% CO_2 for 5-10 minutes. Cells were detached and 5 ml for T25 tissue culture flask of growth media was added to the cells.

Cell suspension was then counted and seeded in a 12-wells plate at the proper dilution (200 cells/mL). One hour after the seeding of the cells, compounds or vehicle were added into the well at the proper concentration (DMSO vehicle 0.1% and 1%; 1 µL of a stock solution 10 mM for each compound to obtain a 10 µM target concentration). For each compound, on the same plates, three technical repetitions were seeded. At least the best 3 independent experiments out of 5 independent repetitions were used to perform the statistical analysis of the results.

After the 12-14 days incubation time, the media was gently removed by aspiration.

Colonies were stained with 350 µL of crystal violet solution and incubated for 30 minutes at room temperature. Crystal violet was removed and plates were washed with PBS and water. The plates were dried.

Colonies were counting using the Gel Count Software.

Digital images of colonies were scanned using a camera. Colonies are counted using the Gel Count Software as described below.

To process plate: open the Gel Count software, Setup Wizard, select 12-wells plate, 2400 dpi, Will.Charm, Acquire only. Once the .ICS was obtained, process it to count the colonies: apply the mask to all the wells, select the red round bottom to circle the colonies, select the proper colonies checking the diameter of colonies and save. Average the three wells for each compounds/vehicle control and divide the mean by the number of cells plated. This is the plate efficiencies (PE):

$$PE = \frac{\text{number of colonies}}{\text{number of cells seeded}} \times 100\%$$

The surviving fraction after irradiation (S) is then calculated as follows:

$$S = \frac{\text{number of colonies}}{\text{number of cells seeded} \times PE}$$

Bibliography

1. Soukupova, J.; Clarkson, R. W. E.; Brancale, A.; Westwell, A. Inhibition of Bcl-3 as a novel therapeutic approach for metastatic breast cancer, **2013**, Ph.D. thesis.

Section 8:

EXPERIMENTAL SECTION

8.1 General information: chemistry

All the chemicals, reagents and solvents were purchased from SIGMA Aldrich or Alfa Aesar without further purification or purified by standard techniques.

Thin Layer Chromatography

Silica gel plates (Merck Kieselgel $60F_{254}$) were used and were developed by the ascending method. After solvent evaporation, compounds were visualised by irradiation with UV light at 254 nm and 366 nm.

Column chromatography

Purification was performed by silica gel chromatography using silica gel 40-60 μm from Merck and the appropriate eluent mixture or using the Interchim PuriFlash 4000 automated column chromatography system.

Melting point

Melting points were determined using Griffin Melting Point Apparatus.

NMR SPECTRA

^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR spectra were recorded using a Bruker AVANCE (500 MHz and 125MHz) spectrometer auto-calibrated to the deuterated solvent reference peak (used the applied solvent simultaneously as internal standard). TMS was used as an internal standard for ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR ($\delta = 0$ ppm).

Chemical shifts (δ) are given in ppm (parts per million) relative to tetramethylsilane (used as internal standard, $\delta = 0$ ppm) together with the relative assignment, the coupling constant ($J_{(H-H)}$ / Hz) and the multiplicity: singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), multiplet (m), broad multiplet (bm).

MASS SPECTROMETRY (MS): Low-resolution mass spectra were performed on Bruker Daltonics microTof-LC in positive or negative mode, atmospheric pressure ionization, electron spray ionization mass spectroscopy (ESI).

HPLC ANALYSIS: All analytical high-performance liquid chromatography (HPLC) experiments were done on a Series 200 UV/Vis Detector provided with a System Controller SN4000, a pump

Spectra System P4000 flow range of 0.1 to 10.0 ml/min and a maximum operating pressure of 6000psi (400 Bar), PerkinElmer Series 200 Column Oven controls, Series 200 UV/Vis Detector using a C18-Varian Pursuit (150 × 4.6 mm, 5 μM) reverse phase column. Samples were prepared by dissolving 1 mg in 5 mL acetonitrile and water solution (1:1), filtered using a 0.2-0.4 u syringe filters, at 254 and 280 nm.

The purity of some final compounds for each scaffold was determined to be >95% by HPLC using the eluents water (eluent A), acetonitrile (eluent B), at two wavelengths (254 nm and 280 nm), under the following conditions: gradient 30 min 10% → 100% eluent B in eluent A (method 1).

8.2 Molecular modelling

All molecular modelling studies were performed on a Mac pro 2.66 GHz Quad-Core Intel Xeon, running Ubuntu.

Molecular Operating Environment (MOE) 2013.08 and Maestro (Schrodinger version) were used as molecular modelling software.

All minimisations were performed with MOE until RMSD gradient of 0.001 Kcal $mol^{-1}Å^{-1}$ with AMBER99 force field. Partial charges were automatically calculated. Conformational analyses were performed with MOE 2013.08.

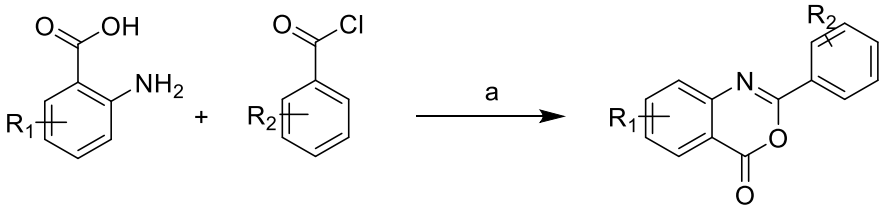
Docking experiments were carried out using LEadIT-FlexX version 2.10, Plants version 1.1 and Glide SP module in Maestro with the default options.

General procedure 1: synthesis of 2-phenyl-4H-benzo[d][1,3]oxazin-4-one intermediates (234 - 296)

A suspension was prepared dissolving 1 equivalent of benzoic acid in pyridine (1 mmol/3 mL) and 2.2 equivalent of the appropriate benzoyl-chloride was added. The reaction mixture was stirred at room temperature over a period between 2 and 12 hours.

The reaction was monitored by TLC and it was stopped after the complete disappearance of the anthranilic acid. The reaction was poured into 10% solution of sodium carbonate. The formed precipitate was collected by filtration under reduced pressure as a powder and washed three times with *n*-hexane in order to obtain the titled compound in high yield.

Scheme[37]

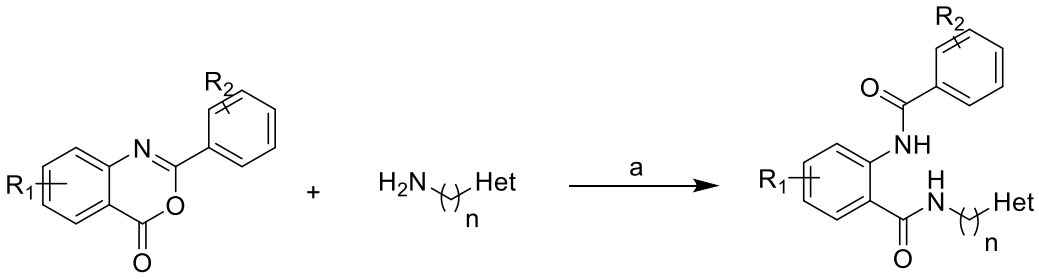


Scheme[37]*. Reagents and conditions:* **a**) pyridine, 25 °C, 2-8 h.

General procedure 2: synthesis of analogues of compound 23 (28-186)

To a solution of 1 equivalent of the starting intermediate in *N*, *N*-dimethyl-formamide was added 2 equivalent of *N*, *N*-diisopropylethylamine and 2.2 equivalent of 2-morpholinoethanamine. The reaction mixture was stirred at room temperature for 4-16 hours. The reaction mixture was diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated to give a crude compound which was purified by silica gel column using a mixture chloroform : methanol (9:1) as eluent. The product was collected under reduced pressure to give the titled powder.

Scheme[38]

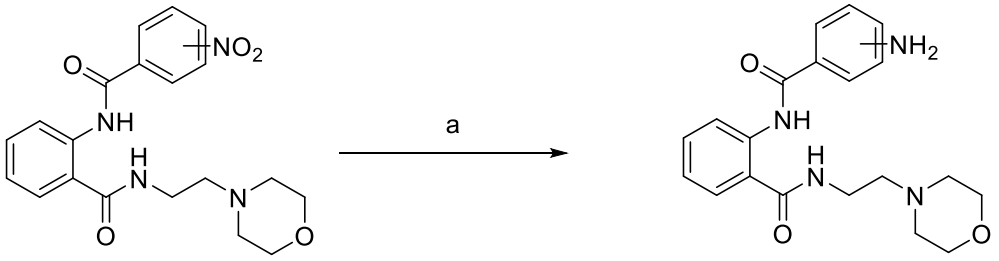


Scheme[38]*. Reagents and conditions:* **a**) DIPEA, DMF, 25 °C, 4 - 16 hours.

General procedure 3: synthesis of 2-(amino-benzamido)-*N*-(2-morpholinoethyl) benzamide compounds (32, 33)

A solution of methanol (1 mL/mmol) and the starting nitro-compound (1 equivalent) and Pd-C (10% w/w of the starting nitrocompound) was stirred under H_2 flow for 24 hours at room temperature. The solution was filtered on a celite bed and the filtrate was evaporated to give a solid which was purified by silica gel column chromatography using chloroform: ethanol (9:1) as eluent. The final compound was re-crystallised in ethanol and water to furnish the final compound.

Scheme[39]

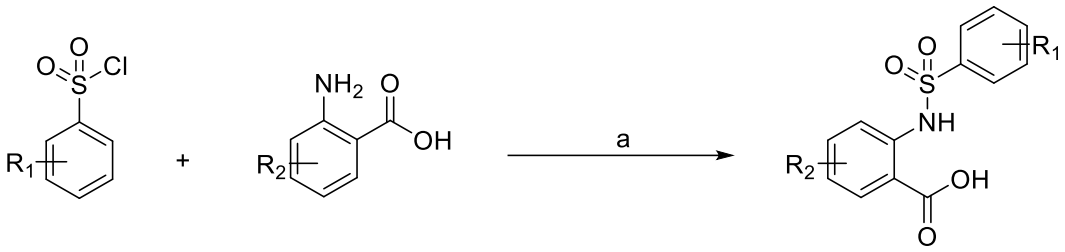


Scheme[39]. *Reagent and conditions:* **a**) H_2, Pd/C, MeOH, 24 h, 25 °C.

General procedure 4: synthesis of 2-(phenyl sulfonamide) benzoic acid intermediates (301-304)

To anthranilic acid (1 equivalent) in water (1mL/mmol) was added sodium hydroxide powder (2 equivalent) and portion-wise cooling down in an ice bath 1 equivalent of sulfonyl chloride. The reaction mixture was stirred for 3-12 hours at room temperature, acidified with HCl 1M and the white precipitate was collected under reduced pressure.

Scheme[40]

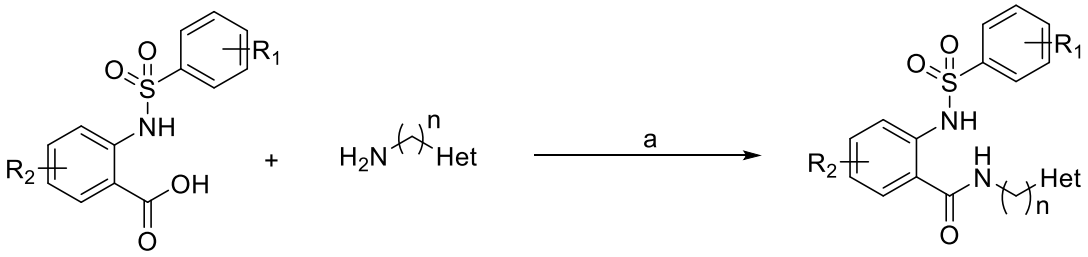


Scheme[40]. *Reagents and reagent condition:* **a**) NaOH, 3-12 hours, 25 °C.

General procedure 5: synthesis of 2-(phenylsulfonamido) benzoic acid intermediates (187-190)

To a mixture of benzoic acid (1 equivalent) in dichloromethane (1mL/mmol) was added EDCI (1.2 equivalent), HOBt (1.2 equivalent) and the mixture was stirred at room temperature for 30 minutes. To this mixture, the primary alkyl amine was added and the reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with water, extracted with dichloromethane, dried over $MgSO_4$, filtered and evaporated to give a yellow oil purified by silica gel column using dichloromethane/methanol as eluent.

Scheme(41)

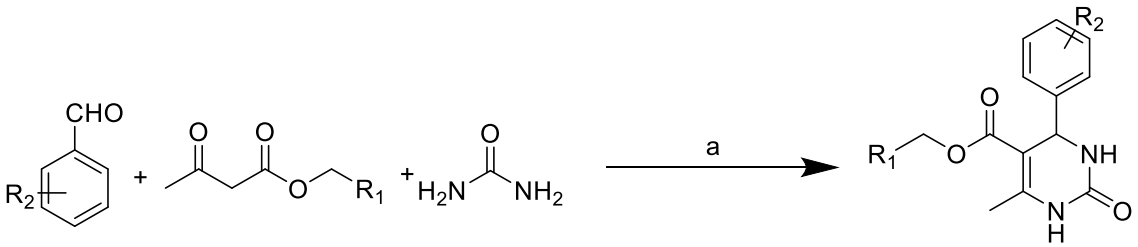


Scheme(41). Reagents and reagent condition: **a**) DIPEA, DMF, 6-16 hours, 25 °C.

General procedure 6: synthesis of benzyl 6-methyl-2-oxo-4-phenyl-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate compounds (191-210)

A solution of 1 equivalent of the suitable benzylacetoacetate, 1 equivalent of the appropriate benzaldehyde and 1.4 equivalent of urea and 5 equivalent of *N*-bromosuccinimide (NBS) in ethanol was heated to reflux temperature for 24 hours. The product precipitated in the reaction mixture and it was re-crystallised, collected under filtration as a powder.

Scheme[42]

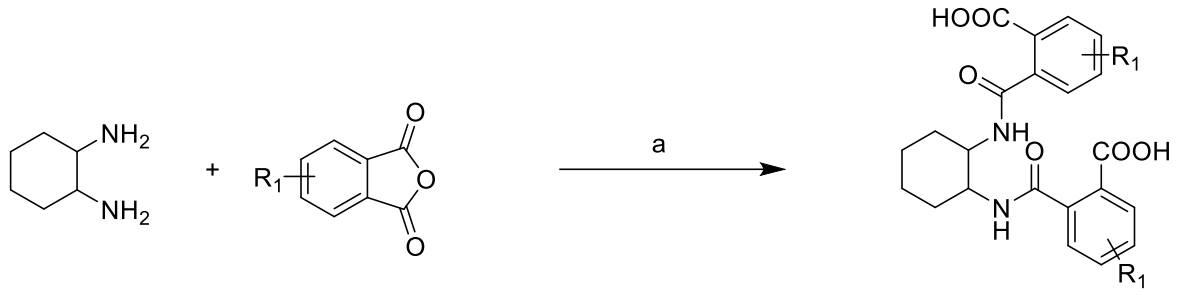


Scheme[42]. *Reagents and conditions:* **a**) NBS, EtOH, 85 °C, 4-24 hours.

General procedure 7 synthesis of the 2, 2'-((cyclohexane-1,2-diylbis(azanediyl))bis(carbonyl))dibenzoic acid compounds (211)

The appropriate phthalic anhydride, 1 equivalent, was added drop-wise to a stirred solution of 1,2-diaminocyclohexane (2 equivalents) in 10% sodium hydroxide. The reaction was stirred at room temperature and the desired compound was collected under filtration as a powder, after acidification with HCl 1M.

Scheme[(43)]

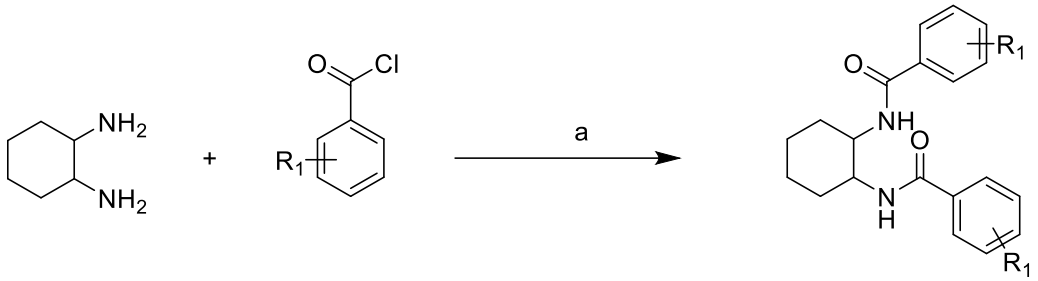


Scheme[(43)].*Reagent and conditions*: **a**) NaOH 10%, 2 hours, 25 °C.

General procedure 8: synthesis of the 2-(amino-benzamido)-*N*-(2-morpholinoethyl)benzamide (212-217)

The appropriate benzoyl chloride, 1 equivalent, was added drop-wise to a stirred solution of 1,2-diaminocyclohexane (2 equivalents) in 10% sodium hydroxide. The reaction was stirred at room temperature and the desired compound was collected under filtration as a powder, purified by silica gel column using ethyl acetate/ *n*-hexane as eluent.

Scheme[44]

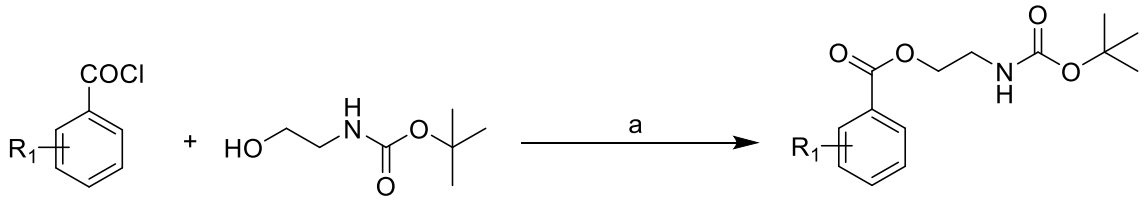


Scheme[44]*. Reagent and conditions:* **a**) NaOH 10%, 30-240 minutes, 25 °C.

General procedure 9: synthesis of the 2-(amino-benzamido)-*N*-(2-morpholinoethyl)benzamide (308 – 310)

A solution of *N*-Boc-ethanolamine (1 equivalent) in sodium hydroxide 1M (3 mL/mmol) was stirred for 15 minutes. The benzoyl chloride (1 equivalent) was added and the reaction mixture was stirred at room temperature for 1-4 hours. The product was collected by filtration under vacuum.

Scheme[45]

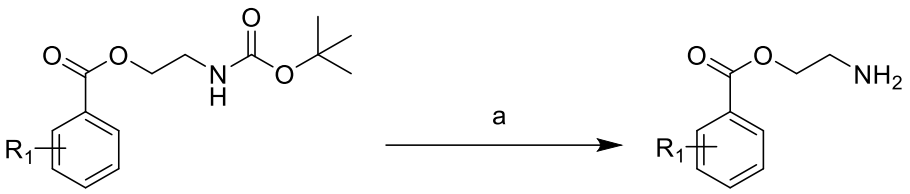


Scheme[45]*. Reagents and reagent condition:* **a**) NaOH 1M, 25° C, 1-4 hours.

General procedure 10: synthesis of the 2-(amino-benzamido)-*N*-(2-morpholinoethyl)benzamide (311 - 313)

A solution of *N*-Boc-ethanolamine (1 equivalent) in trifluoroacetic acid (4 equivalents) was heated at 40 °C for 1-4 hours. The reaction mixture was neutralised using NaOH 1Mm extracted in ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated to give the desired product.

Scheme[46]

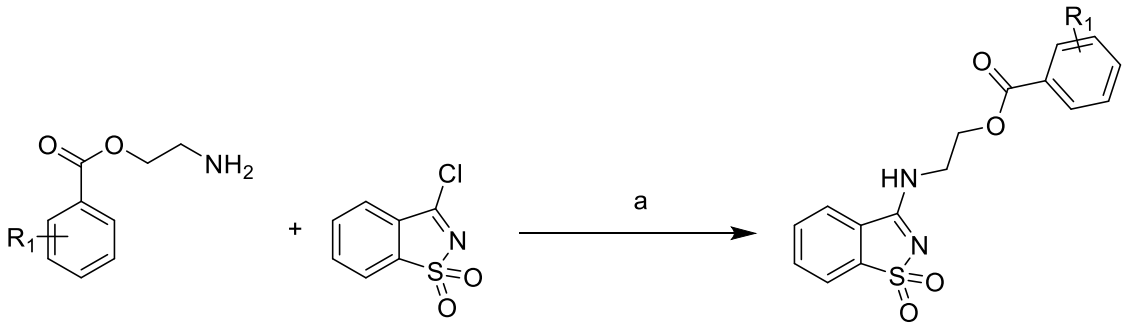


Scheme[46]. *Reagents and reagent condition:* **a**) TFA, DCM, 40° C, 1-4 hours.

General procedure 12: synthesis of the 2-(amino-benzamido)-N-(2-morpholinoethyl)benzamide (219-221)

A solution was prepared by adding sequentially tetrahydrofuran (1.5 mL / 1 mmol), the β-amino-ester (1equivalent), the 3-chloro-1, 2-benzothiazol-1,1-dioxide (1 equivalent), triethylamine (3 equivalent) and it was stirred at room temperature for 12 hours. The solvent was evaporated and the product was purified by silica gel column using ethyl acetate/*n*-hexane as eluent.

Scheme[47]



Scheme[47]. *Reagent and conditions:* **a**) THF, Et_3N, 3-48h, 25 → 60 °C.

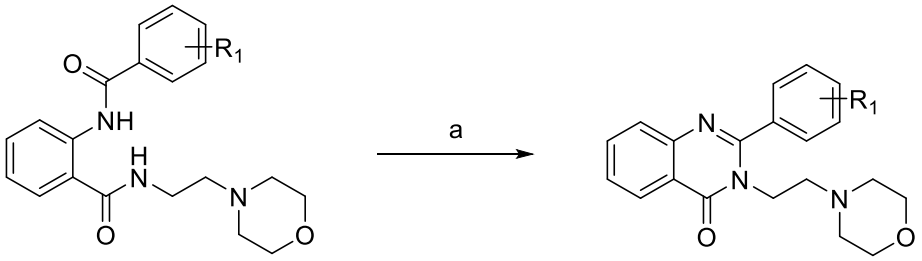
General procedure 6: synthesis of benzyl 6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate compounds (222-225)

A solution was prepared by adding the starting 2-benzamido-N-(2-morpholinoethyl)benzamide (1 equivalent) in formamide (1mL/mmol) in a round bottom flask, which was located in the microwave cavity. The reaction mixture was heated at 170 °C, 15-45 minutes, power max on, 200 W.

The reaction mixture was diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated.

The final compound was then purified by silica gel column chromatography, using dichloromethane : methanol as eluent.

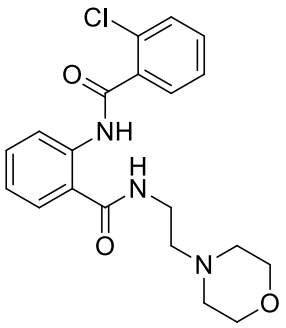
Scheme[48]



Scheme[48]*. Reagents and reagent condition:* **a)** formamide, 210 °C, 15 – 45 minutes.

Synthesis of 2-chloro-N-(2-((2-morpholinoethyl)carbamoyl)phenyl)benzamide (28)

Chemical Formula: $C_{20}H_{22}ClN_3O_3$
Molecular Weight: 387.86



Procedure: 2

State: yellow powder

Yield: 80 %

^{1}H-NMR ($CDCl_3$): δ 2.54 (s, 4H), 2.63 (t, *J* = 5.7 Hz, 2H), 3.52 (dd, *J* = 5.4, 11.0 Hz, 2H), 3.76 (d, *J* = 4.1 Hz, 4H), 6.99 (d, *J* = 18.7 Hz, 1H), 7.20 (t, *J* = 7.6 Hz, 1H), 7.30 – 7.39 (m, 2H), 7.48 (dd, *J* = 1.2, 7.8 Hz, 1H), 7.54 (d, *J* = 7.7 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.68 (dd, *J* = 2.0, 7.2 Hz, 1H), 8.83 (d, *J* = 8.3 Hz, 1H), 11.65 (s, 1H) ppm.

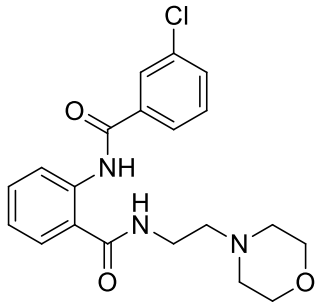
^{13}C-NMR ($CDCl_3$): δ 30.74 (CH_2, C-aliphatic), 35.74 (CH_2, C-aliphatic), 53.18 (CH_2, C-aliphatic), 66.13 (CH_2, C-aliphatic), 120.49 (CH, C-aromatic), 123.37 (CH, C-aromatic), 127.63 (CH, C-aromatic), 128.09 (CH, C-aromatic), 128.89 (CH, C-aromatic), 129.77 (C, C-aromatic), 130.17 (CH, C-aromatic), 131.73 (CH, C-aromatic), 132.02 (CH, C-aromatic), 136.24 (C, C-aromatic), 138.45 (C, C-aromatic), 162.27 (C, C-aromatic), 164.30 (C, C-aromatic), 168.06 (C, C-aromatic) ppm.

MS(ESI)$^+$: 388.1, 389.1 $[M+H]^+$

m.p.: (from ethanol/water) 133-137 °C

Synthesis of 2-(3-chlorobenzamido)-N-(2-morpholinoethyl)benzamide (29)

Chemical Formula: $C_{20}H_{22}ClN_3O_3$
Molecular Weight: 387.86



Procedure: 2

State: white powder

Yield: 60%

^{1}H-NMR ($CDCl_3$): δ 2.55 (s, 4H), 2.66 (t, *J* = 5.9 Hz, 2H), 3.59 (dd, *J* = 5.5, 11.1 Hz, 2H), 3.73 – 3.81 (m, 4H), 7.03 (s, 1H), 7.14 – 7-19 (m, 1H), 7.47 (t, *J* = 7.8 Hz, 1H), 7.52 – 7.56 (m, 2H), 7.57-7.60 (m, 1H), 7.90 – 7.95 (m, 1H), 8.07 (t, *J* = 1.8 Hz, 1H), 8.82 (dd, *J* = 0.7, 8.4 Hz, 1H), 12.35 (s, 1H) ppm.

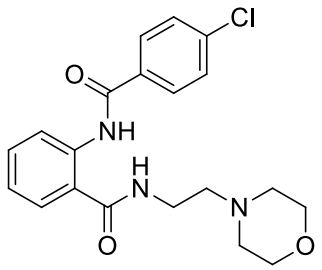
^{13}C-NMR ($CDCl_3$): δ 36.03 (CH_2, C-aliphatic), 53.29 (CH_2, C-aliphatic), 56.55 (CH_2, C-aliphatic), 67.6 (CH_2, C-aliphatic), 120.25 (C, C-aromatic), 121.57 (CH, C-aromatic), 123.15 (CH, C-aromatic), 125.09 (CH, C-aromatic), 1226.64 (CH, C-aromatic), 128.09 (CH, C-aromatic), 128.41 (CH, C-aromatic), 130.05 (CH, C-aromatic), 131.84 (CH, C-aromatic), 132.77 (C, C-aromatic), 135.00 (C, C-aromatic), 136.74 (C, C-aromatic), 139.85 (C, C-aromatic), 164.16 (C, C-aromatic), 169.06 (C, C-aromatic) ppm.

MS(ESI)$^+$: 388.2, 389.2 [M+H]+

m.p.: (from ethanol) 107-109 °C

Synthesis of 2-(2-(4-chlorobenzamido)-N-(2-morpholinoethyl)benzamide (30)

Chemical Formula: $C_{20}H_{22}ClN_3O_3$
Molecular Weight: 387.86



Procedure: 2

State: white powder

Yield: 40 %

^{1}H-NMR ($CDCl_3$): δ 2.54 (s, 4H), 2.66 (t, *J* = 5.9 Hz, 2H), 3.58 (dd, *J* = 5.5, 11.1 Hz, 2H), 3.73 – 3.81 (m, 4H), 7.03 (s, 1H), 7.16 – 7.21 (m, 1H), 7.49 – 7.52 (m, 2H), 7.53 – 7.61 (m, 2H), 7.94 – 8,10 (m, 2H), 8.83 (d, *J* = 8.4 Hz, 1H), 12.35 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.97 (CH_2, C-aliphatic), 53.26 (CH_2, C-aliphatic), 56.43 (CH_2, C-aliphatic), 67.00 (CH_2, C-aliphatic), 120.14 (C, C-aromatic), 121.60 (CH, C-aromatic), 123.05 (CH, C-aromatic), 126.51 (CH, C-aromatic), 128.87 (CH, C-aromatic), 129.03 (CH, C-aromatic), 132.89 (CH, C-aromatic), 133.31 (C, C-aromatic), 138.11 (C, C-aromatic), 140.07 (C, C-aromatic), 164.47 (C, C-aromatic), 169.12(C, C-aromatic) ppm.

MS(ESI)$^+$: 388.1, 389.2 $[M+H]^+$

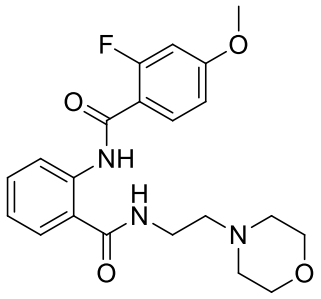
m.p.: (from ethanol/water) 93-95 °C

Elemental analyses:

%Theory			**%Calculated**		
C	**H**	**N**	**C**	**H**	**N**
61.93	5.72	10.83	61.76	5.68	10.62

Synthesis of 2-fluoro-4-methoxy-N-(2-((2-morpholinoethyl)carbamoyl)phenyl)benzamide (31)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 57%

^{1}H-NMR ($CDCl_3$): δ 2.54 (s, 4H), 2.64 (t, *J* = 5.9 Hz, 2H), 3.58 (dd, *J* = 5.4, 11.1 Hz, 2H), 3.73 – 3.78 (m, 4H), 3.88 (s, 3H), 6.71 (dd, *J* = 2.4, 13.3 Hz, 1H), 6.79 – 6.84 (m, 1H), 6.90 (s, 1H), 7.12 – 7.21 (m, 1H), 7.49 – 7.58 (m, 2H), 8.06 (t, *J* = 8.9 Hz, 1H), 8.74 (d, *J* = 8.4 Hz, 1H), 11.69 (d, *J* = 8.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.90 (CH_2, C-aliphatic), 53.28 (CH_2, C-aliphatic), 55.83 (CH_3, C-aliphatic), 56.65 (CH_2, C-aliphatic), 66.89 (CH_2, C-aliphatic), 101.87 (C, C-aromatic), 110.68 (CH, C-aromatic), 114.79 (C, C-aromatic), 122.07 (C, C-aromatic), 122.64 (CH, C-aromatic), 123.19 (CH, C-aromatic), 126.62 (CH, C-aromatic), 132.29 (CH, C-aromatic), 132.90 (CH, C-aromatic), 139.19 (C, C-aromatic), 160.60 (C, C-aromatic), 162.08 (C, C-aromatic), 162.59(C, C-aromatic), 163.67 (C, C-aromatic), 163.76 (C, C-aromatic), 168.72 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.25 ppm.

MS(ESI)$^+$: 402.2 $[M+H]^+$

m.p.: (from ethanol) 115-117 °C

Elemental analyses:

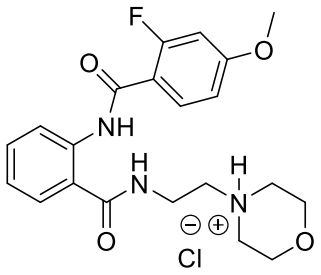
%Theory			%Calculated		
C	**H**	**N**	**C**	**H**	**N**
62.83	6.03	10.46	62.74	6.14	10.12

HPLC (method 1): retention time 11.83 minutes

Synthesis of -(2-(2-(2-fluoro-4-methoxybenzamido)benzamido)ethyl)morpholin-4-ium chloride (31.a)

Chemical Formula: $C_{21}H_{25}ClFN_3O_4$

Molecular weight: 437.90



Procedure: A solution of **26** (1equivalent) was diluted in dichloromethane and HCl 12 M (1 equivalent) was added. The solvent was removed and the crude product was collected under reduced pressure.

Yield: 90 %

State: colourless oil

^{1}H-NMR ($CDCl_3$): δ 2.85 (t, J = 18.7 Hz, 2H), 3.23 (s, 2H), 3.60 (t, J = 17.2 Hz, 2H), 3.79 (s, 3H), 3.99 – 3.83 (m, 4H), 4.17 (t, J = 12.1 Hz, 2H), 6.60 (dd, J = 2.4, 13.3 Hz, 1H), 6.73 (dd, J = 2.4, 8.8 Hz, 1H), 7.04 (dd, J = 14.9, 22.0 Hz, 1H), 7.38 (dd, J = 8.9, 16.3 Hz, 1H), 7.96 (t, J = 8.9 Hz, 1H), 8.34 (t, J = 7.7 Hz, 1H), 8.60 (s, 1H), 8.98 (s, 1H), 11.75 (d, J = 8.1 Hz, 1H), 12.20 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 33.99 (CH_2, C-aliphatic), 53.18 (CH_2, C-aliphatic), 55.83 (CH_3, C-aliphatic), 58.90 (CH_2, C-aliphatic), 63.54 (CH_2, C-aliphatic), 110.56 (CH, C-aromatic), 114.79 (CH, C-aromatic), 120.10 (CH, C-aromatic), 122.14 (C, C-aromatic), 123.53 (CH, C-aromatic), 126.87 (CH, C-aromatic), 132.82 (CH, C-aromatic), 133.09 (CH, C-aromatic), 139.63 (C, C-aromatic), 161.93 (C, C-aromatic), 162.41 (C, C-aromatic), 163.55 (C, C-aromatic), 163.72 (C, C-aromatic), 169.46 (C, C-aromatic) ppm.

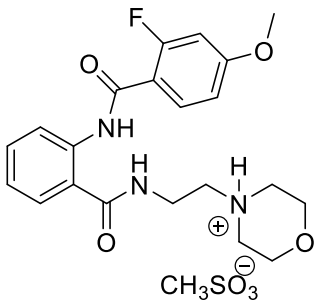
^{19}F-NMR ($CDCl_3$): δ -109.20 ppm.

MS(ESI)$^+$: 402.2 [M + H^+]

Synthesis of 4-(2-(2-(2-fluoro-4-methoxybenzamido)benzamido)ethyl)morpholin-4-ium chloride (31.b)

Chemical Formula: $C_{22}H_{28}FN_3O_7S$

Molecular weight: 497.57



Procedure: A solution of **26** (1equivalent) was diluted in acetone and methane sulfonic acid (1 equivalent) was added. The solvent was removed and the crude product was collected under reduced pressure.

Yield: 90 %

State: yellow oil

^{1}H-NMR ($CDCl_3$): δ 2.37 (s, 3H), 2.51 (dd, J = 3.6, 1.8 Hz, 6H), 3.88 (s, 3H), 3.95 (s, 6H), 6.77 – 7.07 (m, 2H), 7.14 – 7.25 (m, 1H), 7.61 – 7.68 (m, 1H), 7.91 (t, J = 8.9 Hz, 2H), 8.00 – 8.06 (m, 1H), 8.72 (d, J = 7.8 Hz, 1H), 11.89 (d, J = 6.6 Hz, 1H), 13.06-13.32 (bs, 1H) ppm.

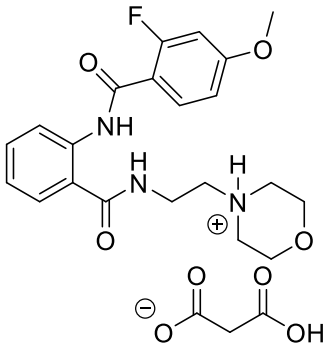
^{13}C-NMR ($CDCl_3$): δ 39.50 (CH_2, C-aliphatic), 40.00 (CH_2, C-aliphatic), 52.22 (CH_2, C-aliphatic), 56.81 (CH_3, C-aliphatic), 58.65 (CH_3, C-aromatic), 110.52 (CH, C-aromatic), 111.90 (CH, C-aromatic), 117.24 (CH, C-aromatic), 120.98 (C, C-aromatic), 123.54 (CH, C-aromatic), 131.69 (CH, C-aromatic), 132.76 (CH, C-aromatic), 134.60 (CH, C-aromatic), 141.19 (C, C-aromatic), 160.75 (C, C-aromatic), 161.86 (C, C-aromatic), 162.16 (C, C-aromatic), 163.82 (C, C-aromatic), 169.25 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.78 ppm.

Synthesis of 4-(2-(2-(2-fluoro-4-methoxybenzamido)benzamido)ethyl)morpholin-4-ium malonic salt (31.c)

Chemical Formula: $C_{24}H_{28}FN_3O_5$

Molecular weight: 505.50



Procedure: A solution of **26** (1equivalent) was diluted in dichloromethane and malonic acid (1 equivalent) was added. The solvent was removed and the crude product was collected under reduced pressure.

Yield: 90 %

State: yellow oil

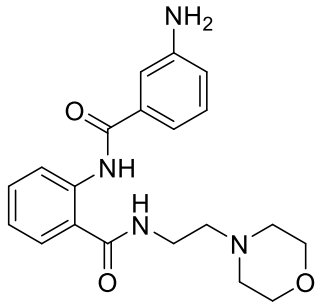
^{1}H-NMR (DMSO-d$_6$): δ 2.52 – 2.59 (m, 1H), 2.60 – 2.70 (m, 5H), 3.13 (d, J = 6.9 Hz, 2H), 3.47 (dd, J = 6.2, 12.2 Hz, 2H), 3.57 – 3.67 (m, 4H), 3.87 (s, 3H), 6.85 – 7.00 (m, 1H), 7.00 – 7.12 (m, 1H), 7.17 – 7.27 (m, 1H), 7.48 – 7.62 (m, 1H), 7.75 (dt, J = 5.8, 11.7 Hz, 1H), 7.83 – 7.94 (m, 1H), 8.56 (d, J = 7.7 Hz, 1H), 8.78 (t, J = 5.1 Hz, 1H), 11.86 (d, J = 6.4 Hz, 1H) ppm.

^{13}C-NMR (DMSO-d$_6$): δ 39.83 (CH_2, C-aliphatic), 41.72 (CH_2, C-aliphatic), 53.21 (CH_2, C-aliphatic), 56.58 (CH_3, C-aliphatic), 57.09 (CH_2, C-aliphatic), 65.93 (CH_2, C-aliphatic), 102.35 (CH, C-aromatic), 111.79 (CH, C-aromatic), 114.97 (C, C-aromatic), 115.07 (CH, C-aromatic), 121.63 (C, C-aromatic), 122.13 (CH, C-aromatic), 123.54 (C, C-aromatic), 128.54 (CH, C-aromatic), 132.35 (CH, C-aromatic), 132.60 (CH, C-aromatic), 139.04 (C, C-aromatic), 160.22 (C, C-aromatic), 161.59 (C, C-aromatic), 162.20 (C, C-aromatic), 168.77 (C, C-aromatic), 169.59 (C, C-aromatic) ppm.

^{19}F-NMR (DMSO-d$_6$): δ -109.78 ppm.

Synthesis of 2-(3-aminobenzamido)-N-(2-morpholinoethyl)benzamide (32)

Chemical Formula: $C_{20}H_{24}N_4O_3$
Molecular Weight: 368.43



Procedure: 3

State: white powder

Yield: 47 %

^{1}H-NMR ($CDCl_3$): δ 2.55 (s, 4H), 2.66 (t, *J* = 5.9 Hz, 2H), 3.58 (dd, *J* = 5.4, 11.1 Hz, 2H), 3.74 – 3.78 (m, 4H), 3.87 (s, 2H), 6.87 (dd, *J* = 1.6, 7.9 Hz, 1H), 7.01 (s, 1H), 7.13 – 7.19 (m, 1H), 7.30 – 7.33 (m, 1H), 7.37 – 7.39 (m, 1H), 7.42 (d, *J* = 7.7 Hz, 1H), 7.55 (dd, *J* = 7.9, 16.7 Hz, 2H), 8.91 – 8.78 (m, 1H), 12.08 (s, 1H) ppm.

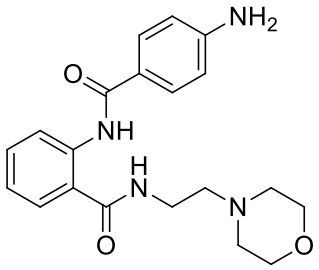
^{13}C-NMR ($CDCl_3$): δ 35.93 (CH_2, C-aromatic), 53.27 (CH_2, C-aromatic), 56.54 (CH_2, C-aromatic), 66.94 (CH_2, C-aromatic), 114.10 (CH, C-aromatic), 117.07 (CH, C-aromatic), 118.35 (CH, C-aromatic), 121.73 (CH, C-aromatic), 122.82 (CH, C-aromatic), 126.53 (CH, C-aromatic), 129.70 (CH, C-aromatic), 132.72 (CH, C-aromatic), 135.75 (C, C-aromatic), 136.51 (C, C-aromatic), 140.09 (C, C-aromatic), 146.80 (C, C-aromatic), 165.67 (C, C-aromatic), 169.18 (C, C-aromatic) ppm.

MS(ESI)$^+$: 369 $[M+H]^+$

m.p.: (from ethanol/water) 126 - 128 °C

Synthesis of 2 2-(4-aminobenzamido)-N-(2-morpholinoethyl)benzamide (33)

Chemical Formula: $C_{20}H_{24}N_4O_3$
Molecular Weight: 368.43



Procedure: 3

State: light brown powder

Yield: 42 %

^{1}H-NMR ($CDCl_3$): δ 2.54 (s, 4H), 2.65 (t, *J* = 5.9 Hz, 2H), 3.51 (s, 4H), 3.55 – 3.61 (m, 1H), 3.73 – 3.78 (m, 1H), 4.05 (s, 2H), 6.75 (d, *J* = 8.6 Hz, 2H), 7.03 (s, 1H), 7.12 (t, *J* = 7.6 Hz, 1H), 7.53 (dd, *J* = 7.8, 13.5 Hz, 2H), 7.90 (d, *J* = 8.5 Hz, 2H), 8.81 (d, *J* = 8.3 Hz, 1H), 11.99 (s, 1H) ppm.

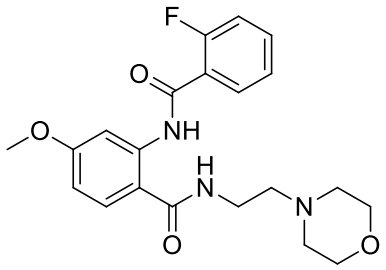
^{13}C-NMR ($CDCl_3$): δ 35.98 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 56.53 (CH_2, C-aliphatic), 66.97 (CH_2, C-aliphatic), 114.32 (CH, C-aromatic), 120.28 (C, C-aromatic), 121.61(CH, C-aromatic), 122.35 (CH, C-aromatic), 124.42 (C, C-aromatic), 126.49 (CH, C-aromatic), 129.33 (CH, C-aromatic), 132.66 (CH, C-aromatic), 140.46 (C, C-aromatic), 149.92 (C, C-aromatic), 165.42 (C, C-aromatic), 169.31 (C, C-aromatic) ppm.

MS(ESI)$^+$: 369.2 $[M+H]^+$

m.p.: (from ethanol/water) 101-105 °C

Synthesis of 2-(2-fluorobenzamido)-4-methoxy-N-(2-morpholinoethyl)benzamide (34)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 36 %

^{1}H-NMR ($CDCl_3$): δ 2.53 (s, 4H), 2.63 (t, *J* = 5.9 Hz, 2H), 3.54 (dd, *J* = 5.4, 11.1 Hz, 2H), 3.73 – 3.78 (m, 4H), 3.94 (s, 3H), 6.71 (dd, *J* = 2.6, 8.7 Hz, 1H), 6.80 (s, 1H), 7.22 (dd, *J* = 8.4, 10.5 Hz, 1H), 7.29 – 7.32 (m, 1H), 7.45 (d, *J* = 8.8 Hz, 1H), 7.48 – 7.53 (m, 1H), 7.95 - 8.04 (m, 1H), 8.55 (d, *J* = 2.6 Hz, 1H), δ 12.33 (d, *J* = 5.3 Hz, 1H) ppm.

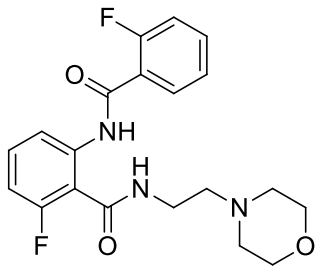
^{13}C-NMR ($CDCl_3$): δ 35.83 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 55.58 (CH_3, C-aliphatic), 56.61 (CH_2, C-aliphatic), 67.00 (CH_2, C-aliphatic), 106.06 (CH, C-aromatic), 110.35 (CH, C-aromatic), 116.62 (C, C-aromatic), 124.60 (CH, C-aromatic), 127.87 (CH, C-aromatic), 131.29 (CH, C-aromatic), 133.31 (CH, C-aromatic),141.66 (CH, C-aromatic) ppm.
^{19}F-NMR (CDCl3): δ -112.57 ppm.
MS(ESI)$^+$: 402.2 $[M+H]^+$

m.p.: (from ethanol/water) 114-116°C
Elemental analyses:

%Theory			**%Calculated**		
C	**H**	**N**	**C**	**H**	**N**
62.83	6.03	10.46	60.72	5.96	9.91

Synthesis of 2-fluoro-6-(2-fluorobenzamido)-N-(2-morpholinoethyl)benzamide (35)

Chemical Formula: $C_{20}H_{21}F_2N_3O_3$
Molecular Weight: 389.40



Procedure: 2

State: white powder

Yield: 23 %

^{1}H-NMR ($CDCl_3$): δ 2.53 (s, 4H), 2.62 (t, *J* = 6.0 Hz, 2H), 3.59 (dd, *J* = 5.0, 10.6 Hz, 2H), 3.72 – 3.81 (m, 4H), 6.93 (dd, *J* = 8.3, 11.8 Hz, 1H), 7.22 (dd, *J* = 8.4, 11.2 Hz, 1H), 7.30 – 7.33 (m, 1H), 7.40 – 7.56 (m, 3H), 8.05 (td, *J* = 7.7, 1.8 Hz, 1H), 8.61 (d, *J* = 8.5 Hz, 1H), 12.08 (d, *J* = 6.4 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.19 (CH_2, C-aliphatic), 53.14 (CH_2, C-aliphatic), 56.05 (CH_2, C-aliphatic), 67.00 (CH_2, C-aliphatic), 110.30 (C, C-aromatic), 110.85 (CH, C-aromatic), 116.59 (CH, C-aromatic), 118.35 (CH, C-aromatic), 124.65 (C, C-aromatic), 131.53 (CH, C-aromatic), 132.59 (CH, C-aromatic), 132.68 (CH, C-aromatic), 133.46 (CH, C-aromatic), 133.53 (CH, C-aromatic), 140.89 (C, C-aromatic), 159.55 (C, C-aromatic), 161.42(C, C-aromatic), 162.45 (C, C-aromatic), 164.64 (C, C-aromatic) ppm.

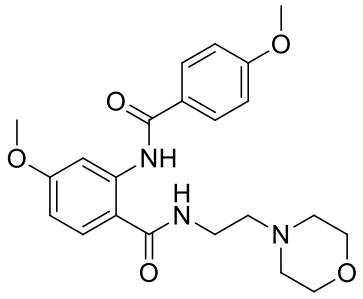
^{19}F-NMR ($CDCl_3$): δ -112.34, -113.94 ppm.

MS(ESI)$^+$:390.2 $[M+H]^+$

m.p.: 88-90°C

Synthesis of 4-methoxy-2-(4-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (36)

Chemical Formula: $C_{22}H_{27}N_3O_5$
Molecular Weight: 413.47



Procedure: 2

State: white powder

Yield: 27 %

^{1}H-NMR ($CDCl_3$): δ 2.60 (s, 4H), 2.70 (s, 2H), 3.59 (d, *J* = 5.2 Hz, 2H), 3.80 (s, 4H), 3.90 (s, 3H), 3.93 (s, 3H), 6.68 (dd, *J* = 2.6, 8.8 Hz, 1H), 6.93 – 7.07 (m, 3H), 7.50 (d, *J* = 8.9 Hz, 1H), 7.94 – 8.10 (m, 2H), 8.60 (d, *J* = 2.6 Hz, 1H), 12.66 (s, 1H) ppm.

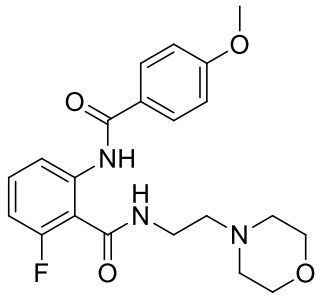
^{13}C-NMR ($CDCl_3$): δ 35.85 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 55.45 (CH_3, C-aliphatic), 55.57(CH_3, C-aliphatic), 56.58 (CH_2, C-aliphatic), 66.99 (CH_2, C-aliphatic), 104.84 (CH, C-aromatic), 109.97 (CH, C-aromatic), 111.90 (C, C-aromatic), 114.00 (CH, C-aromatic), 127.25 (C, C-aromatic), 127.83 (CH, C-aromatic), 129.32 (CH, C-aromatic), 142.85 (C, C-aromatic), 162.51 (C, C-aromatic), 163.01 (C, C-aromatic), 165.45 (C, C-aromatic), 169.10 (C, C-aromatic) ppm.

MS(ESI)$^+$:414.2 $[M+H]^+$

m.p.: (from ethanol/water) 86-88 °C

Synthesis of 2-fluoro-6-(4-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (37)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 51 %

^{1}H-NMR ($CDCl_3$): δ 2.54 (s, 4H), 2.64 (t, *J* = 6.0 Hz, 2H), 3.61 (dd, *J* = 4.8, 10.5 Hz, 2H), 3.79 – 3.72 (m, 4H), 3.90 (s, 3H), 6.80 – 6.88 (m, 1H), 7.07 – 6.96 (m, 2H), 7.40 – 7.48 (m, 1H), 7.62 (d, *J* = 11.8 Hz, 1H), 8.10 – 7.94 (m, 2H), 8.69 (d, *J* = 8.5 Hz, 1H), 12.54 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.24 (CH_2, C-aliphatic), 53.14 (CH_2, C-aliphatic), 55.46 (CH_3, C-aliphatic), 55.98 (CH_2, C-aliphatic), 67.02 (CH_2, C-aliphatic), 109.89 (CH, C-aromatic), 110.09 (CH, C-aromatic), 113.99 (CH, C-aromatic), 117.38 (CH, C-aromatic), 117.40 (C, C-aromatic), 127.07 (CH, C-aromatic), 129.40 (CH, C-aromatic), 132.95 (C, C-aromatic), 133.04 (C, C-aromatic), 142.26 (C, C-aromatic), 160.02 (C, C-aromatic), 161.97 (C, C-aromatic), 162.59 (C, C-aromatic), 165.24 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.56 ppm.

MS(ESI)$^{+}$: 402 $[M+H]^{+}$

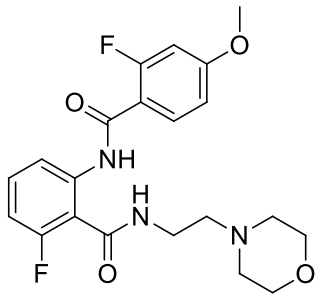
m.p.: (from ethanol/water) 82-84°C

Elemental analyses:

%Theory			%Calculated		
C	**H**	**N**	**C**	**H**	**N**
62.83	6.03	10.46	62.93	6.27	10.40

Synthesis of 2-fluoro-N-(3-fluoro-2-((2-morpholinoethyl)carbamoyl)phenyl)-4-methoxybenzamide (38)

Chemical Formula: $C_{21}H_{23}F_2N_3O_4$
Molecular Weight: 419.42



Procedure: 2

State: white powder

Yield: 40 %

^{1}H-NMR ($CDCl_3$): δ 2.53 (s, 4H), 2.62 (t, *J* = 6.0 Hz, 2H), 3.60 (dd, *J* = 0.6, 5.21 Hz, 2H), 3.73 – 3.77 (m, 4H), 3.88 (d, *J* = 6.1 Hz, 3H), 6.71 (dd, *J* = 2.3, 13.3 Hz, 1H), 6.83 (dd, *J* = 2.3, 8.8 Hz, 1H), 6.91 (dd, *J* = 8.6, 11.3 Hz, 1H), 7.38 (s, 1H), 7.46 (dd, *J* = 8.3, 15.0 Hz, 1H), 8.04 (t, *J* = 8.9 Hz, 1H), 8.56 (d, *J* = 8.5 Hz, 1H), 11.89 (d, *J* = 7.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.19 (CH_2, C-aliphatic), 53.14 (CH_2, C-aliphatic), 55.84 (CH_3, C-aliphatic), 56.08 (CH_2, C-aliphatic), 67.00 (CH_2, C-aliphatic), 101.79 (CH, C-aromatic), 102.00 (CH, C-aromatic), 110.47 (CH, C-aromatic), 110.67 (CH, C-aromatic), 110.73 (CH, C-aromatic), 110.76 (CH, C-aromatic), 118.43(CH, C-aromatic), 132.44 (CH, C-aromatic), 132.53 (CH, C-aromatic), 132.87(CH, C-aromatic), 132.90 (CH, C-aromatic), 140.98 (C, C-aromatic), 158.96 (C, C-aromatic), 160.52 (C, C-aromatic), 161.51 (C, C-aromatic), 162.09 (C, C-aromatic), 162.39 (C, C-aromatic), 163.65 (C, C-aromatic), 164.83 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.44, -112.34 ppm.

MS(ESI)$^+$: 420.2 $[M+H]^+$

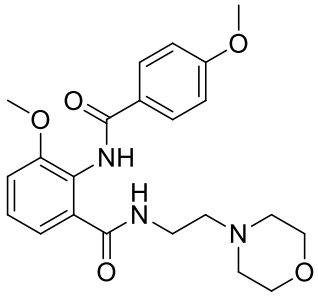
m.p.: 105-107 °C

Elemental analyses:

%Theory			%Calculated		
C	**H**	**N**	**C**	**H**	**N**
60.14	5.53	10.01	60.38	5.53	9.45

Synthesis of 3-methoxy-2-(4-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (39)

Chemical Formula: $C_{22}H_{27}N_3O_5$
Molecular Weight: 413.47



Procedure: 2

State: white powder

Yield: 41 %

^{1}H-NMR ($CDCl_3$): δ 2.43 (s, 4H), 2.51 (t, *J* = 6.0 Hz, 2H), 3.47 (dd, *J* = 5.7, 11.4 Hz, 2H), 3.69 – 3.65 (m, 4H), 3.89 (s, 3H), 3.90 (s, 3H), 6.87 (s, 1H), 6.97 –7.00 (m, 2H), 7.08 (d, *J* = 7.9 Hz, 1H), 7.16 (dd, *J* = 1.1, 7.8 Hz, 1H), 7.24 – 7.31 (m, 1H), 7.92 – 7.97 (m, 2H), 8.89 (s, 1H) ppm.

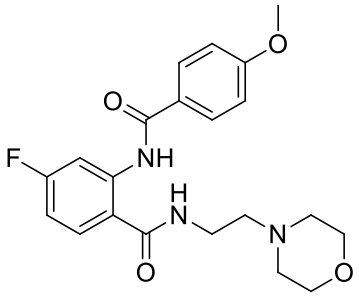
^{13}C-NMR ($CDCl_3$): δ 35.99 (CH_2, C-aromatic), 53.24 (CH_2, C-aromatic), 55.49 (CH_3, C-aromatic), 56.21 (CH_3, C-aromatic), 56.78 (CH_2, C-aromatic), 66.88 (CH_2, C-aromatic), 113.70 (CH, C-aromatic), 113.85 (CH, C-aromatic), 119.42 (CH, C-aromatic), 124.97 (C, C-aromatic), 126.49 (C, C-aromatic), 126.71 (CH, C-aromatic), 129.57 (CH, C-aromatic), 132.25 (C, C-aromatic), 154.07 (C, C-aromatic), 162.61 (C, C-aromatic), 165.76 (C, C-aromatic), 168.45 (C, C-aromatic) ppm.

MS(ESI)$^+$: 414.2 $[M+H]^+$

m.p.: (from ethanol/water) 133-135°C

Synthesis of 4-fluoro-2-(4-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (40)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 23 %

^{1}H-NMR ($CDCl_3$): δ 2.55 (s, 4H), 2.66 (t, *J* = 6.0 Hz, 2H), 3.57 (dd, *J* = 5.6, 11.0 Hz, 2H), 3.74 – 3.78 (m, 4H), 3.90 (s, 3H), 6.79 – 6.81 (m, 1H), 6.96 (s, 1H), 7.00 – 7.06 (m, 2H), 7.51 (dd, *J* = 6.0, 8.7 Hz, 1H), 8.01 – 8.05 (m, 2H), 8.66 – 8.71 (m, 1H), 12.42 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.49 (CH_2, C-aromatic), 41.09 (CH_2, C-aromatic), 55. 46 (CH_3, C-aromatic), 108.69 (CH, C-aromatic), 109.58 (CH, C-aromatic), 114.06 (CH, C-aromatic), 126.81 (C, C-aromatic), 128.77 (CH, C-aromatic), 129.39 (CH, C-aromatic), 138.47 (C, C-aromatic), 142.54 (C, C-aromatic), 162.73 (C, C-aromatic), 165.29 (C, C-aromatic), 165.99 (C, C-aromatic), 168.60, 168.60 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -104.27 ppm.

MS(ESI)$^+$: 402.2 $[M+H]^+$

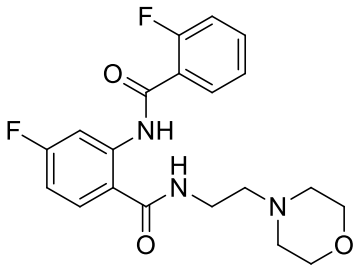
m.p.: (from ethanol/water) 114 – 116 °C

Elemental analyses:

%Theory			%Calculated		
C	**H**	**N**	**C**	**H**	**N**
62.83	6.03	10.46	62.59	6.20	10.26

Synthesis of 4-fluoro-2-(2-fluorobenzamido)-N-(2-morpholinoethyl)benzamide (41)

Chemical Formula: $C_{20}H_{21}F_2N_3O_3$
Molecular Weight: 389.40



Procedure: 2

State: white powder

Yield: 47 %

^{1}H-NMR ($CDCl_3$): δ 2.53 (s, 4H), 2.57 – 2.71 (m, 2H), 3.56 (dd, *J* = 5.5, 11.1 Hz, 2H), 3.69 – 3.85 (m, 4H), 6.79 - 6.86 (m, 2H), 7.16 - 7.22 (m, 1H), 7.25 - 7.31 (m, 1H), 7.45 – 7.58 (m, 2H), 7.98 - 8.06 (m, 1H), 8.65 (dd, *J* = 2.6, 11.9 Hz, 1H), 12.11 (d, *J* = 6.7 Hz, 1H) ppm.

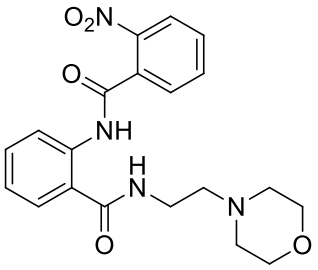
^{13}C-NMR ($CDCl_3$): δ 35.97 (CH_2, C-aromatic), 53.28 (CH_2, C-aromatic), 56.53 (CH_2, C-aromatic), 66.97 (CH_2, C-aromatic), 110.19 (CH, C-aromatic), 110.37 (CH, C-aromatic), 116.50 (CH, C-aromatic), 117.53 (C, C-aromatic), 122.04 (C, C-aromatic), 124.73 (CH, C-aromatic), 128.42 (CH, C-aromatic), 131.57 (CH, C-aromatic), 133.69 (CH, C-aromatic), 159.33 (C, C-aromatic), 161.35 (C, C-aromatic), 163.72 (C, C-aromatic), 165.71 (C, C-aromatic), 168.01 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -104.11, -112.36 ppm.

MS(ESI)$^+$: 390.2 $[M+H]^+$
m.p.: 104 – 106 ° C
HPLC (method 1): retention time 12.8 minutes.

Synthesis of N-(2-morpholinoethyl)-2-(3-nitrobenzamido)benzamide (42)

Chemical Formula: $C_{20}H_{22}N_4O_5$
Molecular Weight: 398.41



Procedure: 2

State: white powder

Yield: 43%

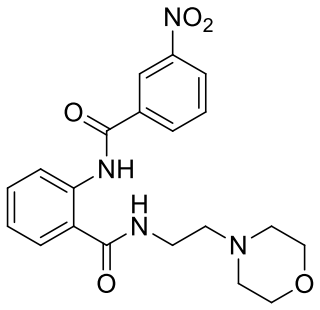
^{1}H-NMR ($CDCl_3$): δ 2.39 (t, *J* = 4.7 Hz, 4H), 2.46 (t, *J* = 6.8 Hz, 2H), 3.37(q, *J* =6.5Hz, 2H), 3.53(t, *J* = 4.6 Hz, 4H), 7.20 - 7.27 (m, 1H), 7.59 (t, *J* = 8.2Hz, 1H), 7.82 (m, 3H), 7.91 (t, *J* = 15.2 Hz, 1H), 8.13 (d, *J* = 8.3, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.48 (CH_2, C-aliphatic), 53.19 (CH_2, C-aliphatic), 56.95 (CH_2, C-aliphatic), 66.14 (CH_2, C-aliphatic), 120.89 (CH, C-aromatic), 121.76 (C, C-aromatic), 123.67(CH, C-aromatic), 124.57 (CH, C-aromatic), 128.11 (CH, C-aromatic), 128.38 (CH, C-aromatic), 131.68 (CH, C-aromatic), 131.58 (CH, C-aromatic), 132.02 (C, C-aromatic), 134.11(CH, C-aromatic), 138.23 (C, C-aromatic), 147.02 (C, C-aromatic), 163.35 (C, C-aromatic), 168.01 (C, C-aromatic) ppm.
MS(ESI)$^+$: 399.19 $[M+H]^+$

m.p.: 138-140 °C

Synthesis of N-(2-morpholinoethyl)-2-(3-nitrobenzamido)benzamide (43)

Chemical Formula: $C_{20}H_{22}N_4O_5$
Molecular Weight: 398.41



Procedure: 2

State: white powder

Yield: 51%

^{1}H-NMR ($CDCl_3$): δ 2.60 (s, 4H), 2.71 (s, 2H), 3.61 (d, *J* = 5.2 Hz, 2H), 3.80 (s, 4H), 7.24 (t, *J* = 7.2 Hz, 2H), 7.61 (t, *J* = 7.4 Hz, 2H), 8.24 (d, *J* = 8.8 Hz, 2H), 8.39 (d, *J* = 8.7 Hz, 2H), 8.86 (d, *J* = 8.1 Hz, 1H), 12.71 (s, 1H) ppm.

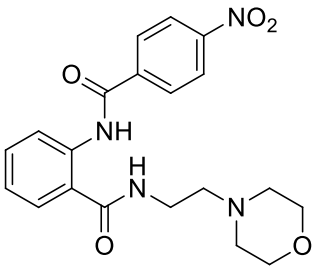
^{13}C-NMR ($CDCl_3$): δ 36.51 (CH_2, C-aliphatic), 53.20 (CH_2, C-aliphatic), 56.93 (CH_2, C-aliphatic), 66.16 (CH_2, C-aliphatic), 120.64 (C, C-aromatic), 120.91 (CH, C-aromatic), 123.42 (CH, C-aromatic), 126.87 (CH, C-aromatic), 127.59 (CH, C-aromatic), 128.12 (CH, C-aromatic), 128.44 (CH, C-aromatic), 132.20 (CH, C-aromatic), 138.76 (CH, C-aromatic), 140.15 (C, C-aromatic), 149.43 (C, C-aromatic), 162.69 (C, C-aromatic), 168.38 (C, C-aromatic), 168.98 (C, C-aromatic) ppm.

MS(ESI)$^+$: 399.2 $[M+H]^+$

m.p.: 147 – 149 °C

Synthesis of N-(2-morpholinoethyl)-2-(2-nitrobenzamido)benzamide (44)

Chemical Formula: $C_{20}H_{22}N_4O_5$
Molecular Weight: 398.41



Procedure: 2

State: white powder

Yield: 51%

^{1}H-NMR ($CDCl_3$): δ 2.44 (m, 6H). 3.44 (q, *J* = 6.5 Hz, 2H), 3.54 (t, *J* = 4.6 Hz, 4H), 7.20 - 7.27 (m, 1H), 7.57 - 7.60 (m, 1H), 7.85 (dd, *J* = 1.5, 8.0 Hz,1H), 8.16 (d, *J* = 8.9 Hz, 2H), 8.44 (d, *J* = 8.9Hz, 2H), 8.59 (d, *J* = 8.4Hz,1H), 8.83(s, 1H), 12.70 (s, 1H) ppm.

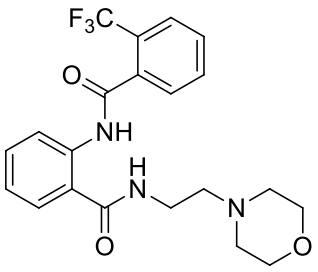
^{13}C-NMR ($CDCl_3$): δ 36.58 (CH_2, C-aliphatic), 53.20 (CH_2, C-aliphatic), 56.93 (CH_2, C-aliphatic), 66.18 (CH_2, C-aliphatic), 120.64 (CH, C-aromatic), 120.94 (C, C-aromatic), 123.41 (CH, C-aromatic), 124.13 (CH, C-aromatic), 128.15 (CH, C-aromatic), 128.43 (CH, C-aromatic), 132.21 (CH, C-aromatic), 138.70 (C, C-aromatic), 140.18 (C, C-aromatic), 149.43 (C, C-aromatic), 162.69 (C, C-aromatic), 168.36 (CH, C-aromatic) ppm.

MS(ESI)$^+$: 399.2 $[M+H]^+$

m.p.: 147 – 149 °C

Synthesis of N-(2-morpholinoethyl)-2-(2-(trifluoromethyl)benzamido)benzamide (45)

Chemical Formula: $C_{21}H_{22}F_3N_3O_3$
Molecular Weight: 421.41



Procedure: 2

State: white powder

Yield: 59 %

^{1}H-NMR ($CDCl_3$): δ 2.50 – 2.55 (m, 4H), 2.60 (dd, *J* = 10.2, 16.1 Hz, 2H), 3.50 (dd, *J* = 5.6, 11.1 Hz, 2H), 3.69 – 3.80 (m, 4H), 6.94 (s, 1H), 7.11 - 7.24 (m, 1H), 7.52 (dd, *J* = 1.4, 7.9 Hz, 1H), 7.56 – 7.62 (m, 2H), 7.66 (t, *J* = 7.4 Hz, 1H), 7.70 (t, *J* = 7.4 Hz, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 8.80 (d, *J* = 8.3 Hz, 1H), δ 11.59 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ 35.90 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 56.48 (CH_2, C-aliphatic), 66.98 (CH_2, C-aliphatic), 120.69 (C, C-aromatic), 121.82 (CH, C-aromatic), 123.46 (CH, C-aromatic), 124.64 (C, C-aromatic), 126.74 (CH, C-aromatic), 126.78 (C, C-aromatic), 127.80 (CH, C-aromatic), 128.15 (CH, C-aromatic), 130.01 (CH, C-aromatic), 132.17(CH, C-aromatic), 132.79 (CH, C-aromatic), 139.46 (C, C-aromatic), 166.14 (C, C-aromatic), 168.71 (C, C-aromatic) ppm.

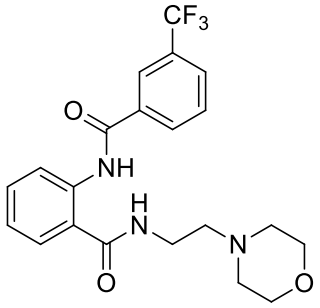
^{19}F-NMR ($CDCl_3$): δ -58.94 ppm.

MS (ESI)$^+$: 422.3 $[M+H]^+$

m.p.: 90-92 °C

Synthesis of N-(2-morpholinoethyl)-2-(3-(trifluoromethyl)benzamido)benzamide (46)

Chemical Formula: $C_{21}H_{22}F_3N_3O_3$
Molecular Weight: 421.41



Procedure: 2

State: white powder

Yield: 63 %

^{1}H-NMR ($CDCl_3$): δ 2.53 – 2.57 (m, 4H), 2.64 – 2.68 (m, 2H), 3.50 – 3.63 (m, 2H), 3.74 – 3.82 (m, 4H), 7.02 (s, 1H), 7.16 -7.26 (m, 1H), 7.54 – 7.62 (m, 2H), 7.67 (d, *J* = 7.8 Hz, 1H), 7.82 (d, *J* = 7.8 Hz, 1H), 8.22 (d, *J* = 7.9 Hz, 1H), 8.38 (s, 1H), 8.85 (dd, *J* = 0.9, 8.4 Hz, 1H), 12.51 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ 36.00 (CH_2, C-aliphatic), 53. 29 (CH_2, C-aliphatic), 56.46 (CH_2, C-aliphatic), 67.00 (CH_2, C-aliphatic), 120.18 (C, C-aromatic), 121.67 (CH, C-aromatic), 123.24 (C, C-aromatic), 125.04 (CH, C-aromatic), 125.07 (CH, C-aromatic), 126.47 (CH, C-aromatic), 128.30, 128.33 (CH, C-aromatic), 129.34 (CH, C-aromatic), 130.09 (CH, C-aromatic), 132.25 (C, C-aromatic), 132.92 (CH, C-aromatic), 135.85 (C, C-aromatic), 139.98 (C, C-aromatic), 164.04 (C, C-aromatic), 169.05 (C, C-aromatic) ppm.

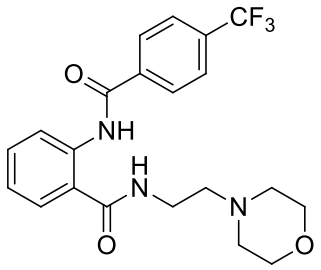
^{19}F-NMR ($CDCl_3$): δ -62.90. ppm.

MS (ESI)$^+$: 422.1 $[M+H]^+$

m.p.: (from ethanol/water) 104-106 °C

Synthesis of N-(2-morpholinoethyl)-2-(4-(trifluoromethyl)benzamido)benzamide (47)

Chemical Formula: $C_{21}H_{22}F_3N_3O_3$
Molecular Weight: 421.41



Procedure: 2

State: white powder

Yield: 22 %

^{1}H-NMR ($CDCl_3$): δ 2.56 (s, 4H), 2.67 (t, *J* = 5.9 Hz, 2H), 3.50 – 3.60 (m, 2H), 3.71 – 3.79 (m, 4H), 7.09 (s, 1H), 7.14 - 7.21 (m, 1H), 7.55 – 7.62(m, 2H), 7.80 (d, *J* = 8.2 Hz, 2H), 8.18 (d, *J* = 8.1 Hz, 2H), 8.86 (dd, *J* = 0.8, 8.4 Hz, 1H), 12.53 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ35.98 (CH_2, C-aliphatic), 53.28 (CH_2, C-aliphatic), 56.46 (CH_2, C-aliphatic), 66.96 (CH_2, C-aliphatic),120.18 (C, C-aromatic), 121.62 (CH, C-aromatic), 123.28 (CH, C-aromatic), 125.82 (CH, C-aromatic), 126.51(CH, C-aromatic), 127.87 (CH, C-aromatic), 132.95 (CH, C-aromatic), 137.85 (C, C-aromatic), 138.22 (C, C-aromatic), 139.99 (C, C-aromatic), 163.66 (C, C-aromatic), 168.94 (C, C-aromatic) ppm.

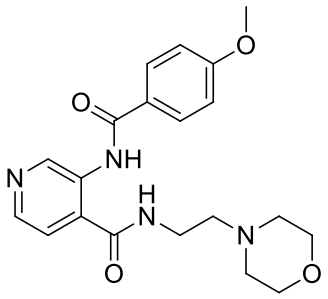
^{19}F-NMR ($CDCl_3$): δ - 63.08 ppm.

MS (ESI)$^+$: 422.1 $[M+H]^+$

m.p.: 99 – 101 °C

Synthesis of 3-(4-methoxybenzamido)-N-(2-morpholinoethyl) wasonicotinamide (48)

Chemical Formula: $C_{20}H_{24}N_4O_4$
Molecular Weight: 384.43



Procedure: 2

State: white powder

Yield: 43 %

^{1}H-NMR ($CDCl_3$): δ 2.55 (s, 4H), 2.67 (t, *J* = 5.9 Hz, 2H), 3.59 (s, 2H), 3.75 – 3.79 (m, 4H), 3.91 (s, 3H), 7.02 – 7.05 (m, 2H), 7.21 (s, 1H), 7.34 (d, *J* = 5.0 Hz, 1H), 8.01 – 8.04 (m, 2H), 8.46 (d, *J* = 5.1 Hz, 1H), 10.12 (s, 1H), 11.80 (s, 1H) ppm.

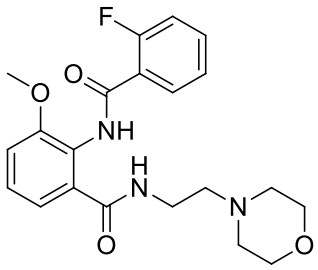
^{13}C-NMR ($CDCl_3$): δ 36.01 (CH_2, C-aromatic), 53.26 (CH_2, C-aromatic), 55.50 (CH_3, C-aromatic), 56.27 (CH_2, C-aromatic), 66.95 (CH_2, C-aromatic), 114.08 (CH, C-aromatic), 119.25 (CH, C-aromatic), 125.67 (C, C-aromatic), 126.31 (C, C-aromatic), 129.48 (CH, C-aromatic), 135.65 (C, C-aromatic), 143.85 (CH, C-aromatic), 144.61 (CH, C-aromatic), 162.81 (C, C-aromatic), 164.94 (C, C-aromatic), 167.32 (C, C-aromatic) ppm.

MS (ESI)$^+$: 395.1 $[M+H]^+$

m.p.: 90 – 92 °C

Synthesis of 2-(2-fluorobenzamido)-3-methoxy-N-(2-morpholinoethyl)benzamide (49)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 31 %

^{1}H-NMR ($CDCl_3$): δ 2.37-2.40 (m, 4H), 2.50 - 2.52 (m, 2H), 3.48-3.51 (m, 2H), 3.62 - 3.65 (m, 4H), 3.91 (s, 3H), 6.77 - 6.79 (m, 1H), 7.08 - 7.09 (m, 1H), 7.19-7.23 (m, 2H), 7.30 - 7.33 (m, 2H), 7.52-7.57 (m, 1H), 8.14 - 8.17 (m, 1H), 8.97 - 8.99 (m, 1H) ppm.

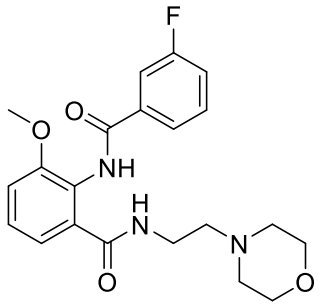
^{13}C-NMR ($CDCl_3$): δ 36.07 (CH_2, C-aliphatic), 53.28 (CH_2, C-aliphatic), 56.20 (CH_3, C-aliphatic), 56.90 (CH_2, C-aliphatic), 66.87 (CH_2, C-aliphatic), 113.26 (CH, C- aromatic), 116.33 (CH, C-aromatic), 119.79 (CH-C- aromatic), 123.60 (C, C- aromatic), 124.76 (CH, C- aromatic), 127.26 (CH, C- aromatic), 132.26 (CH, C- aromatic), 133.77 (CH, C- aromatic), 134.19 (CH, C-aromatic), 138.90 (C, C-aromatic), 153.82 (C, C- aromatic), 159.93 (C, C- aromatic), 161.91 (C, C- aromatic), 168.07 (C, C- aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.47 ppm.

MS(ESI)$^{+}$: 402.2 $[M+H]^{+}$
m.p.: 105 - 107 °C

Synthesis of 2-(3-fluorobenzamido)-3-methoxy-N-(2-morpholinoethyl)benzamide (50)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 88 %

^{1}H-NMR ($CDCl_3$): δ 2.42 - 2.50 (m, 4H), 2.52 - 2.56 (m, 2H), 3.45 - 3.49 (m, 2H), 3.67 - 3.69 (m, 4H), 3.91 (s, 3H), 6.77 - 6.81 (m, 1H), 7.11 - 7.15 (m, 2H), 7.25 - 7.27 (m, 1H), 7.30 - 7.32 (m, 1H), 7.46 - 7.50 (m, 1H), 7.68 - 7.70 (m, 1H), 7.75 (d, *J* = 7.55 Hz, 1H), 9.157 (d, *J* = 7.42, 1H) ppm.

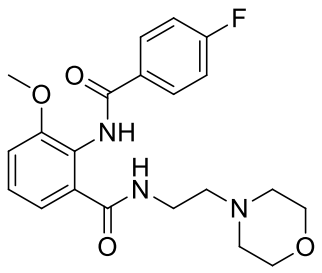
^{13}C-NMR ($CDCl_3$): δ 36.00 (CH_2, C-aliphatic), 53.26 (CH_2, C-aliphatic), 56.24 (CH_3, C-aliphatic), 56.67 (CH_2, C-aliphatic), 66.92 (CH_2, C-aliphatic), 113.6 (CH, C-aromatic), 114.02 (CH, C-aromatic), 115.06 (CH, C- aromatic), 118.99 (CH, C- aromatic), 120.10 (CH, C-aromatic), 123.14 (CH, C- aromatic), 124.83 (C, C- aromatic), 126.96 (CH, C- aromatic), 130.34 (C, C- aromatic), 137.11 (C, C-aromatic), 154.17 (C, C- aromatic), 163.0 (C, C-aromatic), 168.38 (C, C- aromatic), 170.30 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): -111.75 ppm.

MS(ESI)$^{+}$: 402.2 $[M+H]^{+}$
m.p.: 128-131°C

Synthesis of 2-(2-fluorobenzamido)-3-methoxy-N-(2-morpholinoethyl)benzamide (51)

Chemical Formula: $C_{21}H_{24}FN_3O_4$
Molecular Weight: 401.43



Procedure: 2

State: white powder

Yield: 30 %

^{1}H-NMR ($CDCl_3$): δ 2.45 (t, *J* = 4.36 Hz, 4H), 2.537 (dd, *J* = 4.18, 7.84 Hz, 2H), 3.45-3.49 (m, 2H), 3.687 (t, *J* = 4.63 Hz, 4H), 3.913 (s, 3H), 6.79-6.80 (m, 1H), 7.10-7.19 (m, 3H), 7.291 (q, *J* = 4.88 Hz, 2H), 7.98-8.02 (m, 2H), 9.101 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.04 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 56.23 (CH_3, C-aliphatic), 56.71 (CH_2, C-aliphatic), 66.91 (CH_2, C-aliphatic), 114.02 (CH, C- aromatic), 115.59 (CH, C-aromatic), 115.77 (CH, C- aromatic), 119.13 (CH, C- aromatic), 126.83 (CH, C- aromatic), 130.03 (CH, C- aromatic), 130.10 (CH, C- aromatic), 130.49 (C, C- aromatic), 131.78 (C, C- aromatic), 154.23 (C, C- aromatic), 164.05 (C, C- aromatic), 164.92 (C, C- aromatic), 166.06 (C, C-aromatic), 168.42 (C, C- aromatic) ppm.

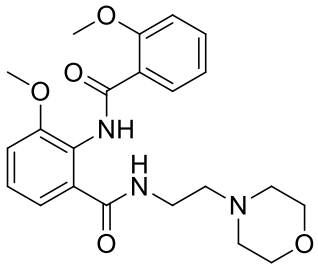
^{19}F-NMR ($CDCl_3$): δ -107.695 ppm.

MS(ESI)$^+$: 402.2 $[M+H]^+$

m.p.: 148-151 °C

Synthesis 3-methoxy-2-(2-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (52)

Chemical Formula: $C_{22}H_{27}N_3O_5$
Molecular Weight: 413.47



Procedure: 2

State: white powder

Yield: 41 %

^{1}H-NMR ($CDCl_3$): δ 2.34 (d, *J* = 14.65 Hz, 4H), 2.46 (t, *J* = 6.06 Hz, 2H), 3.48 (q, *J* = 5.71 Hz, 2H), 3.58 (t, *J* = 4.41 Hz, 4H), 3.90 (s, 3H), 4.07 (s, 3H), 6.87-6.89 (m, 1H), 7.04-7.13 (m, 3H), 7.23 (dd, *J* = 0.78, 7.58 Hz, 1H), 7.30-7.33 (m, 1H), 7.50-7.54 (m, 1H), 8.263 (dd, *J* = 1.48, 7.73 Hz, 1H), 9.89 (s, 1H) ppm.

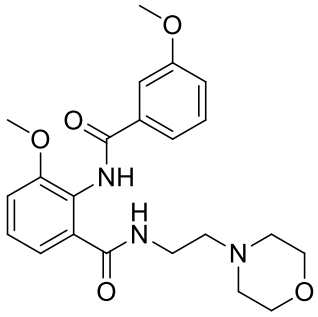
^{13}C-NMR ($CDCl_3$): δ 36.12 (CH_2, C-aliphatic), 53.25 (CH_2, C-aliphatic), 56.20 (CH3, C-aliphatic), 56.97 (CH_2, C-aliphatic), 66.83 (CH_2, C-aliphatic), 111.67 (CH, C- aromatic), 112.82 (CH, C- aromatic), 120.23 (CH, C- aromatic), 121.35 (CH, C- aromatic), 121.35 (C, C- aromatic), 123.56 (C, C- aromatic), 127.06 (CH, C- aromatic), 132.69 (CH, C- aromatic), 133.40 (CH, C- aromatic), 134.77 (C, C- aromatic), 153.73 (C, C- aromatic), 157.91 (C, C- aromatic), 164.56 (C, C- aromatic), 168.28 (C, C-aromatic) ppm.

MS(ESI)$^+$: 414.2 $[M+H]^+$

m.p.: 105-107°C

Synthesis 3-methoxy-2-(3-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (53)

Chemical Formula: $C_{22}H_{27}N_3O_5$
Molecular Weight: 413.47



Procedure: 2

State: white powder

Yield: 37 %

^{1}H-NMR ($CDCl_3$): δ 2.42 (t, *J* = 4.21 Hz, 4H), 2.51 (t, *J* = 6.02 Hz, 2H), 3.47 (q, *J* = 5.69 Hz, 2H), 3.66 (t, *J* = 4.62 Hz, 4H), 3.88 (s, 3H), 3.90 (s, 3H), 6.80 (s, 1H), 7.08-7.12 (m, 2H), 7.159 (dd, *J* = 1.26, 7.77 Hz, 1H), 7.28 -7.31 (m, 1H), 7.39 -7.42 (m, 1H), 7.53 -7.55 (m, 2H), 8.92 (s, 1H) ppm.

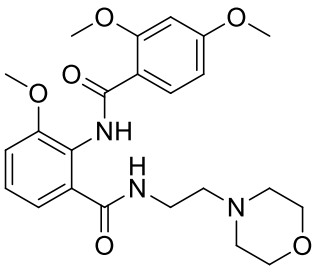
^{13}C-NMR ($CDCl_3$): δ 36.04 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 55.47 (CH_3, C-aliphatic), 56.21 (CH_3, C-aliphatic), 56.78 (CH_2, C-aliphatic), 66.89 (CH_2, C-aliphatic), 112.81 (CH, C-aromatic), 113.75 (CH, C- aromatic), 118.26 (CH, C- aromatic), 119.38 (CH. C- aromatic), 119.58 (CH, C- aromatic), 124.75 (C, C- aromatic), 126.87 (CH, C- aromatic), 126.69 (CH, C-v), 132.35 (C, C- aromatic), 135.69 (C, C- aromatic), 154.12 (C, C- aromatic), 159.89 (C, C- aromatic), 165.96 (C, C- aromatic), 168.32 (C, C- aromatic) ppm.

MS(ESI)$^+$: 414.2 $[M+H]^+$

m.p.: 110- 113°C

Synthesis 3-methoxy-2-(3-methoxybenzamido)-N-(2-morpholinoethyl)benzamide (54)

Chemical Formula: $C_{23}H_{29}N_3O_6$
Molecular Weight: 443.49



Procedure: 2

State: white powder

Yield: 41 %

^{1}H-NMR ($CDCl_3$): δ 2.345 (t, *J* = 4.39 Hz, 4H), 2.454 (t, *J* = 6.14 Hz, 2H), 3.476 (q, *J* = 5.72 Hz, 2H), 3.596 (t, *J* = 4.63 Hz, 4H), 3.892 (d, *J* = 3.17 Hz, 6H), 4.043 (s, 3H), 6.561 (d, *J* = 2.30 Hz, 1H), 6.635 (dd, *J* = 2.32, 8.78 Hz, 1H), 6.933 (s, 1H), 7.035 (dd, *J* = 1.44, 8.11 Hz, 1H), 7.23-7.30 (m, 2H), 8.22 (d, *J* = 8.76 Hz, 1H), 9.72 (s, 1H) ppm.

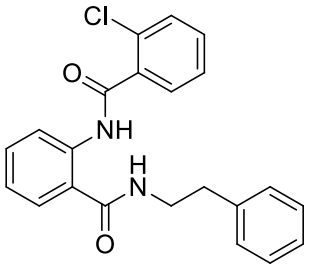
^{13}C-NMR ($CDCl_3$): δ 36.13 (CH_2, C-aliphatic), 53.26 (CH_2, C-aliphatic), 55.60 (CH_3, C-aliphatic), 56.15 (CH_3, C-aliphatic), 56.19 (CH_3, C-aliphatic), 56.99 (CH_2, C-aliphatic), 66.85 (CH_2, C-aliphatic), 98.74 (CH, C- aromatic), 105.51 (CH, C- aromatic), 112.73 (CH, C- aromatic), 114.33 (C, C- aromatic), 120.30 (CH, C- aromatic), 123.69 (C, C- aromatic), 126.95 (CH, C- aromatic), 134.37 (CH, C- aromatic), 134.93 (C, C- aromatic), 153.74 (C, C- aromatic), 159.29 (C, C- aromatic), 163.96 (C, C- aromatic), 164.51 (C, C- aromatic), 168.31 (C, C-aromatic) ppm.

MS(ESI)$^+$: 444.2 $[M+H]^+$

m.p.: 104- 106°C

Synthesis of 2-chloro-N-(2-(phenethylcarbamoyl)phenyl)benzamide (55)

Chemical Formula: $C_{22}H_{19}ClN_2O_2$
Molecular Weight: 378.85



Procedure: 2

State: white powder

Yield: 37%

^{1}H-NMR ($CDCl_3$): δ 2.94 (t, *J* = 6.8 Hz, 4H), 3.70 (dd, *J* = 6.7, 12.9 Hz, 4H), 6.27 (s, 2H), 7.11 (t, *J* = 7.6 Hz, 2H), 7.25 (t, *J* = 8.3 Hz, 5H), 7.32 – 7.44 (m, 10H), 7.49 (dd, *J* = 1.3, 7.8 Hz, 2H), 7.55 (t, *J* = 7.9 Hz, 2H), 7.68 (dd, *J* = 1.8, 7.4 Hz, 2H), 8.79 (d, *J* = 8.4 Hz, 2H), 11.50 (s, 2H) ppm.

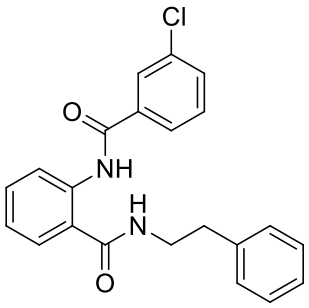
^{13}C-NMR ($CDCl_3$): δ 35.48 (CH_2, C-aliphatic), 41.04 (CH_2, C-aliphatic), 121.80 (C, C-aromatic), 123.37 (CH, C-aromatic), 126.34 (CH, C-aromatic), 126.80 (CH, C-aromatic), 127.14 (CH, C-aromatic), 128.78 (CH, C-aromatic), 128.82 (CH, C-aromatic), 129.32 (CH, C-aromatic), 130.62 (C, C-aromatic), 131.34 (CH, C-aromatic), 132.67 (CH, C-aromatic), 136.16 (C, C-aromatic), 138.49 (C, C-aromatic), 139.18 (C, C-aromatic), 165.41 (C, C-aromatic), 168.76 (C, C-aromatic).

MS(ESI)$^+$: 379.1, 380.1 $[M+H]^+$

m.p.: (from ethanol) 101-103 °C

Synthesis of 2-(3-chlorobenzamido)-N-phenethylbenzamide (56)

Chemical Formula: $C_{22}H_{19}ClN_2O_2$
Molecular Weight: 378.85



Proceure: 2

State: white powder

Yield: 63%

^{1}H-NMR ($CDCl_3$): δ 2.98 (t, *J* = 6.8 Hz, 2H), 3.78 (dd, *J* = 6.7, 12.8 Hz, 2H), 6.31 (s, 1H), 7.07 – 7.13 (m, 1H), 7.23 – 7.30 (m, 3H), 7.33 – 7.38 (m, 3H), 7.48 (t, *J* = 7.8 Hz, 1H), 7.52 – 7.58 (m, 2H), 7.91 (d, *J* = 7.7 Hz, 1H), 8.07 (t, *J* = 1.8 Hz, 1H), 8.79 (d, *J* = 8.4 Hz, 1H), 12.19 (s, 1H) ppm.

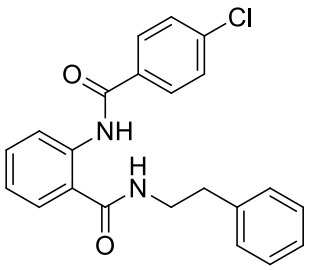
^{13}C-NMR ($CDCl_3$): δ 35.53 (CH_2, C-aliphatic), 41.09 (CH_2, C-aliphatic), 120.42 (C, C-aromatic), 121.66 (CH, C-aromatic), 123.13 (CH, C-aromatic), 125.10 (CH, C-aromatic), 126.30 (CH, C-aromatic), 126.84 (CH, C-aromatic), 128.15 (CH, C-aromatic), 128.80 (CH, C-aromatic), 128.85 (CH, C-aromatic), 130.05 (CH, C-aromatic), 131.87 (CH, C-aromatic), 132.84 (CH, C-aromatic), 135.04 (C, C-aromatic), 136.72 (C, C-aromatic), 138.45 (C, C-aromatic), 139.75 (C, C-aromatic), 164.19 (C, C-aromatic), 169.07 (C, C-aromatic).

MS(ESI)$^+$: 379.1, 380.1 $[M+H]^+$

m.p.: (from ethanol) 103-105 °C

Synthesis of 2-(4-chlorobenzamido)-N-phenethylbenzamide (57)

Chemical Formula: $C_{22}H_{19}ClN_2O_2$
Molecular Weight: 378.85



Procedure: 2

State: white powder

Yield: 61%

^{1}H-NMR ($CDCl_3$): δ 2.98 (t, *J* = 6.8 Hz, 2H), 3.76 (dd, *J* = 6.7, 12.8 Hz, 2H), 6.37 (s, 1H), 7.04 – 7.12 (m, 1H), 7.24 – 7.29 (m, 3H), 7.34 – 7.40 (m, 3H), 7.53 (ddd, *J* = 1.5, 7.6, 8.6 Hz, 3H), 7.97 – 8.02 (m, 2H), 8.79 (d, *J* = 8.4 Hz, 1H), 12.20 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 40.70 (CH_2, C-aliphatic), 42.03 (CH_2, C-aliphatic), 120.30 (CH, C-aromatic), 120.44, 122.99 (CH, C-aromatic), 126.10 (CH, C-aromatic), 128.08 (CH, C-aromatic), 128.25 (CH, C-aromatic), 128.36 (CH, C-aromatic), 128.62 (CH, C-aromatic), 128.82 (CH, C-aromatic), 129.07 (CH, C-aromatic), 132.18 (CH, C-aromatic), 133.27 (C, C-aromatic), 136.89 (C, C-aromatic), 139.05 (C, C-aromatic), 139.11 (C, C-aromatic), 139.26 (C, C-aromatic), 163.27 (C, C-aromatic), 168.62 (C, C-aromatic).

MS(ESI)$^+$: 379.1, 380.1 $[M+H]^+$

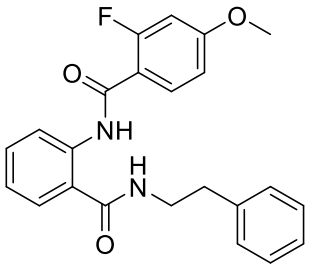
m.p.: 104-106 °C

Elemental analyses:

%Theory			**%Calculated**		
C	**H**	**N**	**C**	**H**	**N**
69.75	5.05	7.39	69.72	5.03	7.33

Synthesis of *N* 2-fluoro-4-methoxy-N-(2-(phenethylcarbamoyl)phenyl)benzamide (58)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: orange powder

Yield: 21 %

^{1}H-NMR ($CDCl_3$): δ 2.97 (t, *J* = 6.8 Hz, 2H), 3.75 (dd, *J* = 6.7, 12.9 Hz, 2H), 3.89 (d, *J* = 4.9 Hz, 3H), 6.17 (s, 1H), 6.73 (dd, *J* = 2.4, 13.3 Hz, 1H), 6.84 (dd, *J* = 2.4, 8.8 Hz, 1H), 7.09 (dd, *J* = 4.2, 10.9 Hz, 1H), 7.26 (d, *J* = 6.1 Hz, 3H), 7.31 – 7.37 (m, 3H), 7.51 (dd, *J* = 4.3, 11.5 Hz, 1H), 8.06 (t, *J* = 8.9 Hz, 1H), 8.71 (d, *J* = 8.4 Hz, 1H), 11.55 (d, *J* = 8.8 Hz, 1H) ppm.

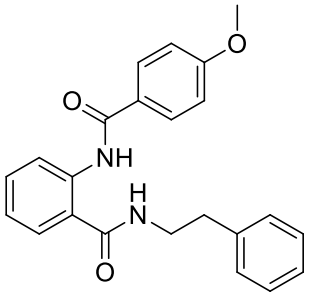
^{13}C-NMR ($CDCl_3$): δ 35.53 (CH_2, C-aliphatic), 41.02 (CH_2, C-aliphatic), 55.84 (CH_3, C-aliphatic), 101.76 (CH, C-aromatic), 101.98 (CH, C-aromatic), 110.72 (CH, C-aromatic), 117.4 (C, C-aromatic), 122.32 (C, C-aromatic), 122.59 (CH, C-aromatic), 123.16 (CH, C-aromatic), 125.10 (C, C-aromatic), 126.37 (CH, C-aromatic), 126.73 (CH, C-aromatic), 128.79 (CH, C-aromatic), 132.25 (CH, C-aromatic), 132.91 (CH, C-aromatic), 138.62 (C, C-aromatic), 138.97 (C, C-aromatic), 158.9 (C, C-aromatic), 163.2 (C, C-aromatic), 168.74 (C, C-aromatic) ppm.
^{19}F-NMR ($CDCl_3$): δ -109.11 ppm.

MS (ESI)$^+$: 415.2 $[M+Na]^+$

m.p.: (from ethanol/water) 139-141 °C
HPLC (method 1): retention time 19.7 minutes.

Synthesis of 2-(4-methoxybenzamido)-*N*-phenethylbenzamide (59)

Chemical Formula: $C_{23}H_{22}N_2O_3$
Molecular Weight: 374.43



Procedure: 2

State: white powder

Yield: 62 %

^{1}H-NMR ($CDCl_3$): δ 2.98 – 3.11 (m, 2H), 3.79 – 3.71 (m, 2H), 3.91 (S, 3H), 6.32 (s, 1H), 7.00-7.08 (m, 3H), 7.20-7.29 (m, 5H), 7.34 (dq, *J* = 7.3, 14.4 Hz, 4H), 7.42 – 7.53 (m, 1H), 7.91 – 8.10 (m, 2H), 8.79 (d, *J* = 8.4 Hz, 1H), 12.01 (s, 1H) ppm.

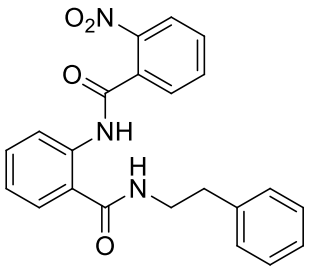
^{13}C-NMR ($CDCl_3$): δ 35.53(CH_2, C-aliphatic), 41.08 (CH_2, C-aliphatic), 55.46 (CH_3, C-aliphatic), 113.98 (CH, C-aromatic), 120.38 (C, C-aromatic), 121.55 (CH, C-aromatic), 122.58 (CH, C-aromatic), 126.31 (CH, C-aromatic), 126.81 (CH, C-aromatic), 127.20 (CH, C-aromatic), 128.82 (CH, C-aromatic), 129.33 (CH, C-aromatic), 132.71 (CH, C-aromatic), 138.52 (C, C-aromatic), 140.15 (C, C-aromatic), 162.51 (C, C-aromatic), 165.71 (C, C-aromatic), 169.26 (C, C-aromatic) ppm.

MS(ESI)$^+$: 375.2 $[M+H]^+$

m.p.: (from ethanol) 124-126 °C

Synthesis of 2-nitro-*N*-(2-(phenethylcarbamoyl)phenyl)-benzamide (60)

Chemical Formula: $C_{22}H_{19}N_3O_4$
Molecular Weight: 389.40



Purification: 2

State: white powder

Yield: 45 %

^{1}H-NMR ($CDCl_3$): δ 2.94 (t, *J* = 6.8 Hz, 2H), 3.69 (dd, *J* = 6.8, 12.8 Hz, 2H), 6.30 (s, 1H), 7.24 – 7.28 (m, 3H), 7.36 (t, *J* = 7.4 Hz, 3H), 7.56 (t, *J* = 7.3 Hz, 1H), 7.58 - 7.67 (m, 1H), 7.70 – 7.79 (m, 3H), 8.07 – 8.12 (m, 1H), 8.72 (d, *J* = 8.3 Hz, 1H), 11.67 (s, 1H) ppm.

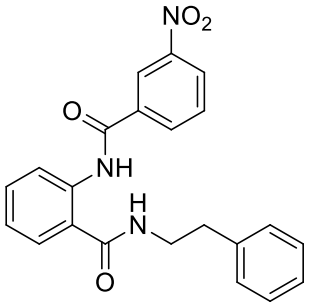
^{13}C-NMR ($CDCl_3$): δ 35.43 (CH_2, C-aliphatic), 41.09 (CH_2, C-aliphatic), 120.63 (C, C-aromatic), 121.98 (CH, C-aromatic), 123.65 (CH, C-aromatic), 124.74 (CH, C-aromatic), 126.28 (CH, C-aromatic), 126.81 (CH, C-aromatic), 128.49 (CH, C-aromatic), 128.80 (CH, C-aromatic), 128.85 (CH, C-aromatic), 130.78 (CH, C-aromatic), 132.88 (CH, C-aromatic), 133.06 (CH, C-aromatic),133.77 (C, C-aromatic), 138.45 (CH, C-aromatic), 139.23 (C, C-aromatic), 147.02 (C, C-aromatic), 164.36 (C, C-aromatic), 168.81 (C, C-aromatic) ppm.

MS(ESI)$^+$: 390.2 $[M+H]^+$

m.p.: (from ethanol/water) 103 - 105 °C

Synthesis of 2-(3-nitrobenzamido)-*N*-phenethylbenzamide (61)

Chemical Formula: $C_{22}H_{19}N_3O_4$
Molecular Weight: 389.40



Procedure: 2

State: white powder

Yield: 14%

^{1}H-NMR ($CDCl_3$): δ 2.94 (t, *J* = 6.8 Hz, 2H), 3.70 (dd, *J* = 6.7, 12.8 Hz, 2H), 6.27 (s, 1H), 7.12 (dd, *J* = 4.2, 11.0 Hz, 1H), 7.25 (t, *J* = 8.4 Hz, 2H), 7.35 (t, *J* = 7.3 Hz, 3H), 7.38 – 7.44 (m, 2H), 7.49 (dd, *J* = 1.2, 7.9 Hz, 1H), 7.52 – 7.57 (m, 1H), 7.68 (dd, *J* = 1.8, 7.4 Hz, 1H), 8.79 (d, *J* = 8.4 Hz, 1H), 11.50 (s, 1H) ppm.

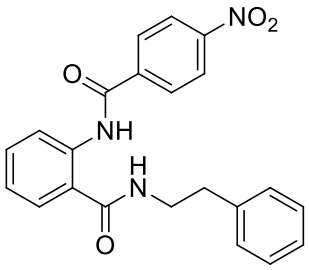
^{13}C-NMR ($CDCl_3$): δ 35.50 (CH_2, C-aliphatic), 41.16 (CH_2, C-aliphatic), 120.14 (C, C-aromatic), 121.59 (CH, C-aromatic), 123.06 (CH, C-aromatic), 123.47 (CH, C-aromatic), 126.26 (CH, C-aromatic), 126.34 (CH, C-aromatic), 126.86 (CH, C-aromatic), 128.80 (CH, C-aromatic), 128.86 (CH, C-aromatic), 129.96 (CH, C-aromatic), 132.76 (CH, C-aromatic), 133.02 (CH, C-aromatic), 136.79 (C, C-aromatic), 138.42 (C, C-aromatic), 139.66 (C, C-aromatic), 148.64 (C, C-aromatic), 163.06 (C, C-aromatic), 169.08 (C, C-aromatic) ppm.

MS(ESI)$^+$: 379.1 $[M+H]^+$

m.p.: (from ethanol) 102-104 °C

Synthesis of 2-(4-nitrobenzamido)-*N*-phenethylbenzamide (62)

Chemical Formula: $C_{22}H_{19}N_3O_4$
Molecular Weight: 389.40



Procedure: 2

State: white powder

Yield: 34%

^{1}H-NMR ($CDCl_3$): δ 2.99 (t, *J* = 6.8 Hz, 4H), 3.78 (dd, *J* = 6.7, 12.8 Hz, 4H), 6.36 (s, 2H), 7.15 (td, *J* = 7.8, 1.1 Hz, 2H), 7.27 (d, *J* = 4.8 Hz, 3H), 7.33 – 7.43 (m, 6H), 7.54 – 7.64 (m, 2H), 8.15 – 8.26 (m, 4H), 8.35 – 8.45 (m, 4H), 8.82 (dd, *J* = 0.8, 8.4 Hz, 2H), 12.54 (s, 2H) ppm.

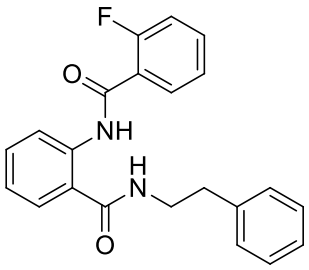
^{13}C-NMR ($CDCl_3$): δ 35.51(CH_2, C-aliphatic), 41.14 (CH_2, C-aliphatic), 120.17 (C, C-aromatic), 121.55 (CH, C-aromatic), 123.54 (CH, C-aromatic), 124.01 (CH, C-aromatic), 126.35 (CH, C-aromatic), 126.90 (CH, C-aromatic), 128.62 (CH, C-aromatic), 128.79 (CH, C-aromatic), 128.88 (CH, C-aromatic), 133.06 (CH, C-aromatic), 138.36 (C, C-aromatic), 139.65 (C, C-aromatic), 140.39 (C, C-aromatic), 150.01 (C, C-aromatic), 162.99 (C, C-aromatic), 168.95 (C, C-aromatic) ppm.

MS(ESI)$^+$: 390.2 $[M+H]^+$

m.p.: (from ethanol/H_2O) 133 - 137 °C

Synthesis of 2-fluoro-*N*-(2-(phenethylcarbamoyl)phenyl)benzamide (63)

Chemical Formula: $C_{22}H_{19}FN_2O_2$
Molecular Weight: 362.40



Procedure: 2

State: white powder

Yield: 40 %

^{1}H-NMR ($CDCl_3$): δ 2.95 (t, *J* = 6.8Hz, 2H), 3.75 (dd, *J* = 6.7, 12.8 Hz, 2H), 6.22 (s, 1H), 7.10 (t, *J* = 7.7 Hz,1H), 7.21-7.25 (m, 3H), 7.28-7.37 (m, 5H), 7.51-7.55 (m, 2H), 7.95 - 8.09 (m, 1H), 8.76 (d, *J* = 8.4 Hz, 1H), 11.67(d, *J* = 7.1 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.52 (CH_2, C-aliphatic), 41.03 (CH_2, C-aliphatic), 116.56 (C, C-aromatic), 122.18 (C, C-aromatic), 122.48 (CH, C-aromatic), 123.39 (CH, C-aromatic), 124.63 (CH, C-aromatic), 124.65 (CH, C-aromatic), 126.39 (CH, C-aromatic), 126.74 (CH, C-aromatic), 128.80 (CH, C-aromatic), 131.58 (CH, C-aromatic), 132.34 (CH, C-aromatic), 133.37(CH, C-aromatic), 133.44 (CH, C-aromatic),138.58 (C, C-aromatic), 138.87 (C, C-aromatic), 168.69 (C, C-aromatic) ppm.

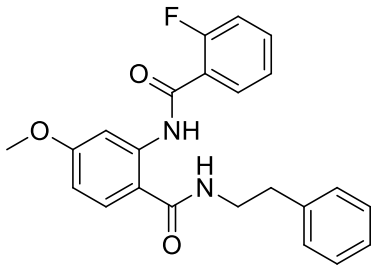
^{19}F-NMR ($CDCl_3$): δ -112.39 ppm.

MS(ESI)+: 385.1 $[M+Na]^+$

m.p.: (from ethanol/water) 95-97 °C

Synthesis of 2-(2-fluorobenzamido)-4-methoxy-N-phenethylbenzamide (64)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: white powder

Yield: 51 %

^{1}H-NMR ($CDCl_3$): δ .2.95 (t, ,*J* =6.71 Hz, 2H), 3.61 (q, *J* =6.71 Hz, 2H), 3.90 (s, 3H), 6.13 (s, 1H), 6.64 (dd, *J* =2.43, 5.80, 1Hz),7.23 (t, *J* =8.42, 5H), 7.32 (q, *J* =8.42, 3H), 7.56-7.52 (m, 1H), 8.00-8.10 (m, 1H), 8.51 (d, *J* =2.50 Hz, 1H), 12.17 (d, *J* =5.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.58 (CH_2, C-aliphatic), 40.93 (CH_2, C-aliphatic), 55.55 (CH_3, C-aliphatic), 106.16 (CH, C-aromatic), 110.23 (CH, C-aromatic), 113.62 (C, C-aromatic), 116.63 (d,$J_{C\text{-}F}$=23.9 Hz, C, C-aromatic), 124.61(CH, C-aromatic), 124.64 (CH, C-aromatic), 126.70 (CH, C-aromatic), 127.70 (CH, C-aromatic), 128.78 (CH, C-aromatic), 128.82 (CH, C-aromatic), 131.31 (CH, C-aromatic), 133.36 (CH, C-aromatic), 138.72(C, C-aromatic), 141.41 (C, C-aromatic), 162.68 (C, C-aromatic) ppm.

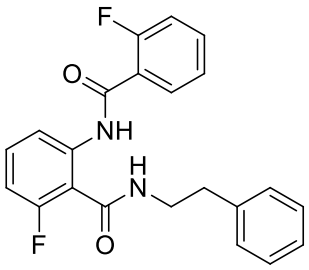
^{19}F-NMR ($CDCl_3$): δ -112.49 ppm.

MS(ESI)$^+$: 415.1 $[M+Na]^+$

m.p.: (from ethanol/water) 108 - 110°C

Synthesis of 2-fluoro-6-(2-fluorobenzamido)-N-phenethylbenzamide (65)

Chemical Formula: $C_{22}H_{18}F_2N_2O_2$
Molecular Weight: 380.39



Procedure: 2

State: white powder

Yield: 48 %

^{1}H-NMR ($CDCl_3$): δ 2.96 (t, *J* = 6.9 Hz, 2H), 3.74 – 3.81 (m, 2H), 6.68 (s, 1H), 6.88 (m, 1H), 7.20 – 7.27 (m, 4H), 7.29 – 7.35 (m, 3H), 7.38 - 7.49 (m, 1H), 7.51 – 7.58 (m, 1H), 7.92 - 8.05 (m, 1H), 8.59 (d, *J* = 8.5 Hz, 1H), 11.96 (d, *J* = 6.3 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.42 (CH_2, C-aliphatic), 41.22 (CH_2, C-aliphatic), 110.70 (C, C-aromatic), 116,61 (CH, C-aromatic), 118.33 (CH, C-aromatic), 122.68 (CH, C-aromatic), 124.67 (CH, C-aromatic), 126.69 (CH, C-aromatic), 128.75 (CH, C-aromatic), 131.54 (CH, C-aromatic), 132.65 (CH, C-aromatic), 133.53 (CH, C-aromatic), 138.47 (C, C-aromatic), 140.80 (C, C-aromatic), 159.37 (C, C-aromatic), 161.35 (C, C-aromatic), 162.44 (C, C-aromatic), 164.70 (C, C-aromatic) ppm.

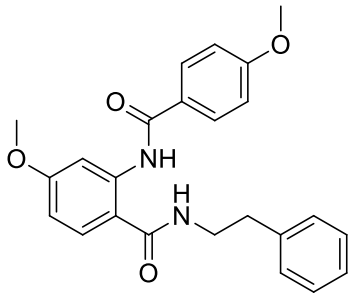
`^{19}F-NMR ($CDCl_3$): δ -111.60, -112.88 ppm.

MS(ESI)$^+$: 381.1 $[M+H]^+$

m.p.: 96-98°C

Synthesis of 4-methoxy-2-(4-methoxybenzamido)-N-phenethylbenzamide (66)

Chemical Formula: $C_{24}H_{24}N_2O_4$
Molecular Weight: 404.46



Procedure: 2

State: white powder

Yield: 34 %

^{1}H-NMR ($CDCl_3$): δ 2.97 (t, *J* = 6.8 Hz, 2H), 3.75 (dd, *J* = 6.7, 12.8 Hz, 2H), 3.89 (s, 6H), 6.13 (d, *J* = 61.2 Hz, 1H), 6.58 (dd, *J* = 2.5, 8.7 Hz, 1H), 7.04 (d, *J* = 8.8 Hz, 2H), 7.27 (d, *J* = 8.3 Hz, 3H), 7.36 (t, *J* = 7.6 Hz, 3H), 8.05 (d, *J* = 8.8 Hz, 2H), 8.57 (d, *J* = 2.5 Hz, 1H), 12.51 (s, 1H) ppm.

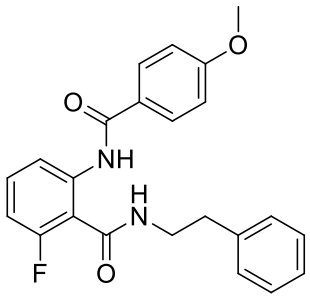
^{13}C-NMR ($CDCl_3$): δ 35.61 (CH_2, C-aliphatic), 40.98 (CH_2, C-aliphatic), 55.46(CH_3, C-aliphatic), 55.55 (CH_3, C-aliphatic), 104.90 (CH, C-aromatic), 109.86 (CH, C-aromatic), 112.07 (C, C-aromatic), 114.01 (CH, C-aromatic), 126.77 (CH, C-aromatic), 127.22 (C, C-aromatic), 127.62 (CH, C-aromatic), 128.81 (CH, C-aromatic), 129.34 (CH, C-aromatic), 138.66 (C, C-aromatic), 142.68 (C, C-aromatic), 162.53 (C, C-aromatic), 162.97 (C, C-aromatic), 165.45(C, C-aromatic),169.10 (C, C-aromatic) ppm.

MS (ESI)$^+$: 405 $[M+H]^+$

m.p.: (from ethanol/water) 138 - 140 °C

Synthesis of 2-fluoro-6-(4-methoxybenzamido)-N-phenethylbenzamide (67)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: white powder

Yield: 45 %

^{1}H-NMR ($CDCl_3$): δ 2.98 (t, *J* = 6.9 Hz, 2H), 3.74 – 3.84 (m, 2H), 3.91 (s, 3H), 6.79 – 6.92 (m, 2H), 7.01 – 7.06 (m, 2H), 7.23 – 7.28 (m, 3H), 7.35 (t, *J* = 7.5 Hz, 2H), 7.32 - 7.45 (m, 1H), 8.01 – 8.05 (m, 2H), 8.68 (d, *J* = 8.5 Hz, 1H), 12.47 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.41 (CH_2, C-aliphatic), 41.26 (CH_2, C-aliphatic), 55.47 (CH_3, C-aliphatic), 109.81 (CH, C-aromatic), 110.01 (CH, C-aromatic), 114.01 (CH, C-aromatic), 117.41 (CH, C-aromatic), 126.76 (CH, C-aromatic), 127.06 (CH, C-aromatic), 128.76 (C, C-aromatic), 129.42 (CH, C-aromatic), 132.99 (CH, C-aromatic), 133.08 (CH, C-aromatic), 138.43 (C, C-aromatic), 159.86 (C, C-aromatic), 161.81 (C, C-aromatic), 162.61 (C, C-aromatic), 165.26 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.18 ppm.

MS (ESI)$^+$: 393 $[M+H]^+$

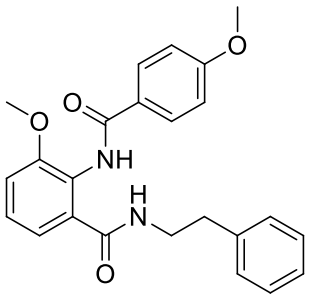
m.p.: (from ethanol/water) 103-105 °C

Elemental analyses:

%Theory			%Calculated		
C	**H**	**N**	**C**	**H**	**N**
70.40	5.39	7.14	70.23	5.71	7.07

Synthesis of 3-methoxy-2-(4-methoxybenzamido)-N-phenethylbenzamide (68)

Chemical Formula: $C_{24}H_{24}N_2O_4$
Molecular Weight: 404.46



Procedure: 2

State: white powder

Yield: 74 %

^{1}H-NMR ($CDCl_3$): δ 2.80 (t, *J* = 7.0 Hz, 2H), 3.61 (dd, *J* = 7.1, 13.2 Hz, 2H), 3.88 (s, 3H), 3.89 (s, 3H), 6.56 (t, *J* = 5.6 Hz, 1H), 6.97– 7.01 (m, 3H), 7.04 (dd, *J* = 2.9, 8.0 Hz, 2H), 7.19 – 7.34 (m, 5H), 7.91 – 7.99 (m, 2H), 8.82 (s, 1H) ppm.

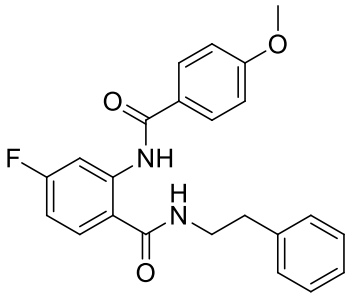
^{13}C-NMR ($CDCl_3$): δ 35.49 (CH_2, C-aromatic), 41.05 (CH_2, C-aromatic), 55.48 (CH_3, C-aromatic), 56.17 (CH_3, C-aromatic), 113.62 (CH, C-aromatic), 113.88 (CH, C-aromatic), 119.28 (CH, C-aromatic), 124.74 (CH, C-aromatic), 126.37 (C, C-aromatic), 126.50 (C, C-aromatic), 126.87 (CH, C-aromatic), 128.62 (CH, C-aromatic), 128.76 (CH, C-aromatic), 129.59 (CH, C-aromatic), 132.75 (C, C-aromatic), 138.81 (C, C-aromatic), 154.18 (C, C-aromatic), 162.61 (C, C-aromatic), 166.12 (C, C-aromatic), 168.42 (C, C-aromatic) ppm.

MS (ESI)$^+$: 405 $[M+H]^+$

m.p.: (from ethanol/water) 136 - 138°C

Synthesis of 4-fluoro-2-(4-methoxybenzamido)-N-phenethylbenzamide (69)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: white powder

Yield: 72 %

^{1}H-NMR ($CDCl_3$): δ 2.97 (d, *J* = 6.8 Hz, 1H), 3.76 (dd, *J* = 6.8, 12.7 Hz, 2H), 3.91 (s, 3H), 6.28 (s, 1H), 6.63 - 6.73 (m, 1H), 7.02 – 7.06 (m, 2H), 7.24 – 7.40 (m, 7H), 7.94– 8.05 (m, 2H), 8.65 (dd, *J* = 2.6, 11.9 Hz, 1H), 12.26 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.49 (CH_2, C-aromatic), 41.09 (CH_2, C-aromatic), 55.46 (CH_3, C-aromatic), 108.47 (CH, C-aromatic), 108.69 (CH, C-aromatic), 109.40 (CH, C-aromatic), 109.58 (C, C-aromatic), 114.06 (CH, C-aromatic), 126.84 (CH, C-aromatic), 128.16 (CH, C-aromatic), 128.77 (CH, C-aromatic), 129.39 (CH, C-aromatic), 138.47 (C, C-aromatic), 142.47 (C, C-aromatic), 162.73 (C, C-aromatic), 164.47 (C, C-aromatic), 165.29 (C, C-aromatic), 165.99 (C, C-aromatic), 168.60 (C, C-aromatic) ppm.

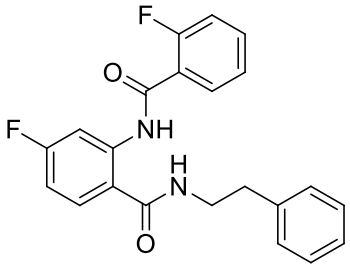
^{19}F-NMR ($CDCl_3$): -104.11 ppm.

MS (ESI)$^+$: 415.1 $[M+Na]^+$

m.p.: (from ethanol/water) 127 - 129 °C

Synthesis of 4-fluoro-2-(2-fluorobenzamido)-N-phenethylbenzamide (70)

Chemical Formula: $C_{22}H_{18}F_2N_2O_2$
Molecular Weight: 380.39



Procedure: 2

State: white powder

Yield: 44 %

^{1}H-NMR ($CDCl_3$): δ 2.96 (t, *J* = 6.8 Hz, 2H), 3.75 (dd, *J* = 6.7, 12.8 Hz, 2H), 6.13 (s, 1H), 6.76-6.79 (m, 1H), 7.23 – 7.27 (m, 4H), 7.30 – 7.38 (m, 4H), 7.47 - 7.61 (m, 1H), 7.91 - 8.07 (m, 1H), 8.63 (dd, *J* = 2.6, 11.8 Hz, 1H), 11.95 (d, *J* = 6.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.47 (CH_2, C-aromatic), 41.06 (CH_2, C-aromatic), 109.49 (CH, C-aromatic), 110.15 (CH, C-aromatic), 116.52 (CH, C-aromatic), 122.02 (CH, C-aromatic), 124.74 (C, C-aromatic), 126.80 (CH, C-aromatic), 128.11 (CH, C-aromatic), 128.19 (CH, C-aromatic), 128.80 (CH, C-aromatic), 128.83 (CH, C-aromatic), 131.62 (C, C-aromatic), 133.65 (CH, C-aromatic), 133.72 (C, C-aromatic), 161.12 (C, C-aromatic), 162.98 (C, C-aromatic), 165.22 (C, C-aromatic), 167.69 (C, C-aromatic), 170.3 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ-103.79, -113.02 ppm.

MS (ESI)$^+$: 403.1 $[M+Na]^+$

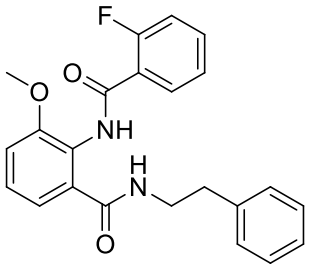
m.p.: (from ethanol/water) 104 – 106 °C

Elemental analyses:

%Theory			**%Calculated**		
C	**H**	**N**	**C**	**H**	**N**
69.47	4.77	7.36	69.51	4.35	7.35

Synthesis of 2-(2-fluorobenzamido)-3-methoxy-N-phenethylbenzamide (71)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: white powder

Yield: 66 %

^{1}H-NMR ($CDCl_3$): δ 2.87 (t, *J* = 7.12 Hz, 2H), 3.65 (dd, *J* = 5.96, 7.10 Hz, 2H), 3.89 (s, 3H), 6.38-6.40 (m, 1H), 7.07 (m, 2H), 7.18-7.23 (m, 4H), 7.26-7.32 (m, 4H), 7.52-7.56 (m, 1H), 8.12-8.15 (m, 1H), 8.83-8.86 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.53 (CH_2, C-aliphatic), 41.02 (CH_2, C-aliphatic), 56.16 (CH_3, C-aliphatic), 113.21 (CH, C-aromatic), 116.32 (CH, C- aromatic), 119.59 (CH, C- aromatic), 123.36 (C, C-aromatic), 124.75 (CH, C- aromatic), 126.42 (CH, C- aromatic), 127.41 (CH, C- aromatic), 128.56 (CH, C- aromatic), 128.73 (CH, C- aromatic), 132.25 (CH, C- aromatic), 133.74 (CH, C- aromatic), 134.06 (C, C- aromatic), 138.91 (C, C- aromatic), 153.95 (C, C- aromatic), 159.92 (C, C- aromatic), 161.90 (C, C- aromatic), 168.10 (C, C- aromatic) ppm.

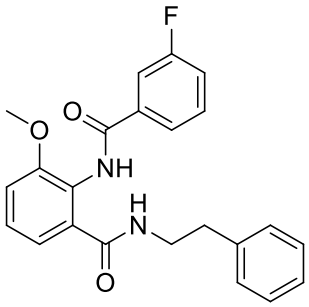
^{19}F-NMR ($CDCl_3$): δ -112.645 ppm.

MS (ESI)$^{+}$: 393.2 $[M+H]^{+}$

m.p.: 130-132 °C

Synthesis of 2-(3-fluorobenzamido)-3-methoxy-N-phenethylbenzamide (72)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: white powder

Yield: 42 %

^{1}H-NMR ($CDCl_3$): δ 2.875 (t, *J* = 7.10 Hz, 2H), 3.62-3.66 (m, 2H), 3.89-3.89 (m, 6H), 6.38-6.39 (m, 1H), 7.02-7.07 (m, 2H), 7.10-7.12 (m, 1H), 7.20-7.26 (m, 4H), 7.28-7.31 (m, 2H), 7.40 (t, *J* = 8.15 Hz, 1H), 7.52-7.53 (m, 2H), 8.81 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.50 (CH_2, C-aliphatic), 41.04 (CH_2, C-aliphatic), 55.47 (CH_3 C-aliphatic), 112.75 (CH, C-aromatic), 113.69 (CH, C- aromatic), 118.33 (CH, C- aromatic), 119.24 (CH, C-aromatic), 119.61 (CH, C- aromatic), 124.54 (C, C- aromatic), 126.50 (CH, C- aromatic), 126.99 (CH, C- aromatic), 128.61 (CH, C- aromatic), 128.74 (CH, C- aromatic), 129.70 (CH, C- aromatic), 132.77 (C, C- aromatic), 135.68 (C, C- aromatic), 138.78 (C, C- aromatic), 154.20 (C, C- aromatic), 159.89 (C, C- aromatic), 166.32 (C, C- aromatic), 168.31 (C, C- aromatic) ppm.

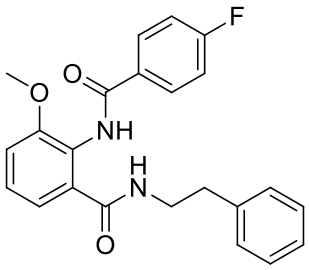
^{19}F-NMR ($CDCl_3$): δ - 111.82 ppm.

MS (ESI)$^+$: 393.2 $[M+H]^+$

m.p.: 99-101 °C

Synthesis of 2-(4-fluorobenzamido)-3-methoxy-N-phenethylbenzamide (73)

Chemical Formula: $C_{23}H_{21}FN_2O_3$
Molecular Weight: 392.42



Procedure: 2

State: white powder

Yield: 36%

^{1}H-NMR ($CDCl_3$): δ 2.47 – 2.38 (m, 4H), 2.60 – 2.49 (m, 2H), 3.47 (dd, *J* = 5.6, 11.4 Hz, 2H), 3.71 – 3.63 (m, 4H), 3.91 (s, 3H), 6.79 (s, 1H), 7.19 – 7.09 (m, 4H), 7.32 – 7.26 (m, 2H), 8.07 – 7.94 (m, 2H), 9.11 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.89 (CH_2, C-aromatic), 40.58 (CH_2, C-aromatic), 56.33 (CH_3, C-aromatic), 113.99 (CH, C-aromatic), 119.30 (CH, C-aromatic), 124.88 (C, C-aromatic), 125.48 (CH, C-aromatic), 126.07 (CH, C-aromatic), 127.04 (CH, C-aromatic), 127.92 (CH, C-aromatic), 129.80 (CH, C-aromatic), 130.39 (C, C-aromatic), 131.66 (CH, C-aromatic), 138.89 (C, C-aromatic), 154.26 (C, C-aromatic), 163.95 (C, C-aromatic), 165.21 (C, C-aromatic), 166.41 (C, C-aromatic), 167.98 (C, C-aromatic) ppm.

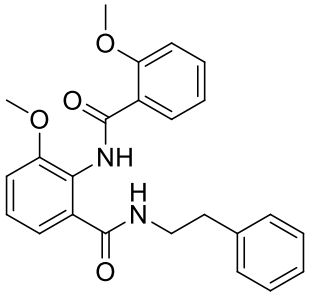
^{19}F-NMR ($CDCl_3$): δ -111.86 ppm.

MS (ESI)$^+$: 415.1$[M+Na]^+$

m.p.: 140-142 °C

Synthesis of 3-methoxy-2-(2-methoxybenzamido)-N-phenethylbenzamide (74)

Chemical Formula: $C_{24}H_{24}N_2O_4$
Molecular Weight: 404.46



Procedure: 2

State: white powder

Yield: 43 %

^{1}H-NMR ($CDCl_3$): δ 2.83 (t, *J* = 7.3 Hz, 2H), 3.65 – 3.59 (m, 2H), 3.88 (s, 3H), 4.06 (s, 3H), 6.65 (s, 1H), 7.04 (dd, *J* = 8.3, 14.5 Hz, 2H), 7.13 (d, *J* = 7.7 Hz, 3H), 7.19 (d, *J* = 7.4 Hz, 3H), 7.27 – 7.22 (m, 2H), 7.55 – 7.50 (m, 1H), 8.25 (t, *J* = 9.1 Hz, 1H), 9.78 – 9.70 (m, 1H),

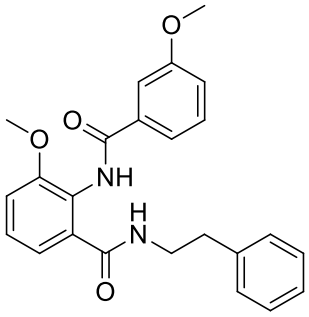
^{13}C-NMR ($CDCl_3$): δ 39.14 (CH_2, C-aliphatic), 40.73 (CH_2, C-aliphatic), 56.24 (CH_3, C-aliphatic), 56.26 (CH_3, C-aliphatic), 111.15 (CH, C-aromatic), 113.01 (CH, C-aromatic), 120.16 (CH, C-aromatic), 121.14 (CH, C-aromatic), 122.99 (C, C-aromatic), 123.30 (C, C-aromatic), 126.36 (CH, C-aromatic), 127.03 (CH, C-aromatic), 128.90 (CH, C-aromatic), 132.26 (CH, C-aromatic), 133.52 (CH, C-aromatic), 135.40 (CH, C-aromatic), 138.82 (C, C-aromatic), 153.73(C, C-aromatic), 157.76 (C, C-aromatic), 164.55 (C, C-aromatic), 167.98 (C, C-aromatic) ppm.

MS (ESI)$^+$: 405.2 $[M+H]^+$

m.p.: (from methanol/water) 134 - 136 °C

Synthesis of 3-methoxy-2-(3-methoxybenzamido)-N-phenethylbenzamide (75)

Chemical Formula: $C_{24}H_{24}N_2O_4$
Molecular Weight: 404.46



Procedure: 2

State: white powder

Yield: 42%

^{1}H-NMR ($CDCl_3$): δ 2.87 (t, *J* = 7.10 Hz, 2H), 3.62-3.66 (m, 2H), 3.89-3.89 (m, 6H), 6.38-6.39 (m, 1H), 7.02-7.07 (m, 2H), 7.10-7.12 (m, 1H), 7.20-7.26 (m, 4H), 7.28-7.31 (m, 2H), 7.40 (t, *J* = 8.15 Hz, 1H), 7.51-7.53 (m, 2H), 8.81 (s, 1H) ppm.

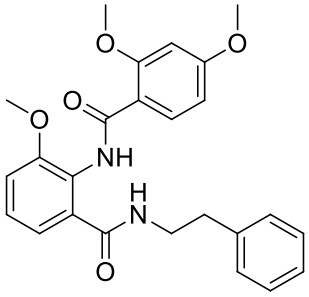
^{13}C-NMR ($CDCl_3$): δ 35.50 (CH, C-aliphatic), 41.04 (CH, C-aliphatic), 55.47 (CH3 C-aliphatic), 56.16 (CH3, C-aliphatic), 112.75 (CH, C-aromatic), 113.69 (CH, C-aromatic), 118.33 (CH, C-aromatic), 119.24 (CH, C-aromatic), 119.61 (CH, C-aromatic), 124.54 (C, C-aromatic), 126.50 (CH, C-aromatic), 126.99 (CH, C-aromatic), 128.61 (CH, C-aromatic), 128.74 (CH, C-aromatic), 129.70 (CH, C-aromatic), 132.77 (C, C-aromatic), 135.68 (C, C-aromatic), 138.78 (C, C-aromatic), 154.20 (C, C-aromatic), 159.89 (C, C-aromatic), 166.32 (C, C-aromatic), 168.31 (C, C-aromatic) ppm.

MS (ESI)$^+$: 405.2 $[M+H]^+$

m.p.: 100-102 °C

Synthesis of 2-(2, 4-dimethoxybenzamido)-3-methoxy-N-phenethylbenzamide (76)

Chemical Formula: $C_{25}H_{26}N_2O_5$
Molecular Weight: 434.48



Procedure: 2

State: white powder

Yield: 68%

^{1}H-NMR ($CDCl_3$): δ 2.81 (t, *J* = 7.33 Hz, 2H), 3.59-3.63 (m, 2H), 3.87 (s, 3H), 3.900 (s, 3H), 4.02 (s, 3H), 6.56 (d, *J* = 2.32 Hz, 1H), 6.64 (dd, *J* = 2.33, 8.80 Hz, 1H), 6.75 (s, 1H), 7.01 (dd, *J* = 1.28, 8.28 Hz, 1H), 7.14-7.20 (m, 4H), 7.24-7.27 (m, 3H), 8.228 (d, *J* = 8.76 Hz, 1H), 9.608 (s, 1H) ppm.

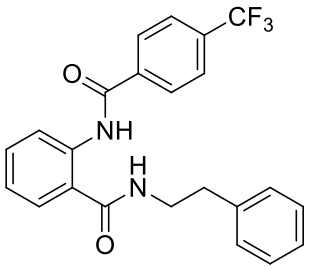
^{13}C-NMR ($CDCl_3$): δ 35.56 (CH_2, C-aliphatic), 41.03 (CH_2, C-aliphatic), 55.58 (CH_3, C-aliphatic), 56.13 (CH_3, C-aliphatic), 56.15 (CH_3, C-aliphatic), 98.75 (CH, C-aromatic), 105.55 (CH, C-aromatic), 112.71 (CH, C- aromatic), 114.27 (C, C- aromatic), 120.13 (CH, C-aromatic), 123.51 (C, C-aromatic), 126.25 (CH, C- aromatic), 127.25 (CH, C- aromatic), 128.45 (CH, C- aromatic), 128.70 (CH, C- aromatic), 128.82 (CH, C- aromatic), 134.38 (CH, C- aromatic), 135.64 (C, C-aromatic), 139.11 (C, C- aromatic), 154.04 (C, C- aromatic), 159.32 (C, C- aromatic), 163.96 (C, C-aromatic), 165.10 (C, C- aromatic), 168.33 (C, C- aromatic) ppm.

MS (ESI)$^+$: 435.2 $[M+H]^+$

m.p.: 132-134 °C

Synthesis of N-phenethyl-2-(4-(trifluoromethyl)benzamido)benzamide (77)

Chemical Formula: $C_{23}H_{19}F_3N_2O_2$
Molecular Weight: 412.40



Procedure: 2

State: white powder

Yield: 53 %

^{1}H-NMR ($CDCl_3$): δ 3.06 – 2.90 (m, 2H), 3.78 (dd, *J* = 6.8, 12.7 Hz, 2H), 6.31 (s, 1H), 6.95 - 7.12 (m, 1H), 7.19 - 7.26 (m, 3H), 7.40 – 7.31 (m, 4H), 7.59 – 7.54 (m, 1H), 7.81 (d, *J* = 8.2 Hz, 2H), 8.18 (d, *J* = 8.1 Hz, 2H), 8.83 (dd, *J* = 0.9, 8.4 Hz, 1H), 12.36 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ 34.84 (CH_2, C-aliphatic), 40.75 (CH_2, C-aliphatic), 119.87 (C, C-aromatic), 121.14 (CH, C-aromatic), 123.01 (CH, C-aromatic), 125.47 (CH, C-aromatic), 126.06 (CH, C-aromatic), 126.74 (CH, C-aromatic), 127.92 (CH, C-aromatic), 128.66 (CH, C-aromatic), 128.90 (CH, C-aromatic), 132.93 (CH, C-aromatic), 138.52 (C, C-aromatic), 140.02 (C, C-aromatic), 148.16 (C, C-aromatic), 157.09 (C, C-aromatic), 163.97 (C, C-aromatic), 188.19 (C, C-aromatic) ppm.

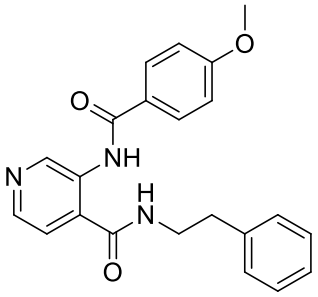
^{19}F-NMR ($CDCl_3$): δ – 63.09 ppm.

MS (ESI)$^+$: 435.1 $[M+H]^+$

m.p.: (from ethanol/water) 96 - 98 °C

Synthesis of 3-(4-methoxybenzamido)-*N*-phenethylisonicotinamide (78)

Formula: $C_{22}H_{21}N_3O_3$
Molecular Weight: 375.42



Procedure: 2

State: white powder

Yield: 34 %

^{1}H-NMR ($CDCl_3$): δ 2.99 (t, *J* = 6.8 Hz, 2H), 3.78 (dd, *J* = 6.8, 12.8 Hz, 2H), 3.91 (s, 3H), 6.73 (s, 1H), 7.03 – 7.06 (m, 2H), 7.14 (d, *J* = 5.0 Hz, 1H), 7.21 – 7.27 (m, 2H), 7.25 - 7.34 (m, 3H), 7.99 – 8.02 (m, 2H), 8.32 (d, *J* = 5.1 Hz, 1H), 10.04 (s, 1H), 11.63 (s, 1H) ppm.

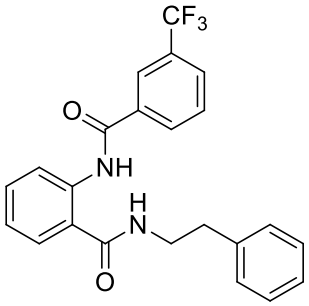
^{13}C-NMR ($CDCl_3$): δ 35.34 (CH_2, C-aromatic), 41.21 (CH_2, C-aromatic), 55.51 (CH_3, C-aromatic), 114.11 (CH, C-aromatic), 119.23 (CH, C-aromatic), 126.04 (C, C-aromatic), 126.23 (C, C-aromatic), 126.94 (CH, C-aromatic), 128.49 (CH, C-aromatic), 128.78 (CH, C-aromatic), 128.89 (CH, C-aromatic), 129.50 (CH, C-aromatic), 135.37 (C, C-aromatic), 138.23 (C, C-aromatic), 143.80 (CH, C-aromatic), 144.46 (CH, C-aromatic), 162.85 (C, C-aromatic), 164.99 (C, C-aromatic), 167.35 (C, C-aromatic) ppm.

MS(ESI)$^+$: 376 $[M+H]^+$

m.p.: (from ethanol/water) 120 - 122°C

Synthesis of N-phenethyl-2-(3-(trifluoromethyl)benzamido)benzamide (79)

Chemical Formula: $C_{23}H_{19}F_3N_2O_2$
Molecular Weight: 412.40



Procedure: 2

State: white powder

Yield: 51 %

^{1}H-NMR ($CDCl_3$): δ 2.98 (t, *J* = 6.8 Hz, 2H), 3.78 (dd, *J* = 6.7, 12.8 Hz, 2H), 6.32 (s, 1H), 7.12 (t, *J* = 7.6 Hz, 1H), 7.26 (d, *J* = 7.4 Hz, 3H), 7.34 – 7.39 (m, 3H), 7.59 – 7.53 (m, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.83 (d, *J* = 7.7 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.38 (s, 1H), 8.81 (d, *J* = 8.4 Hz, 1H), 12.35 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ 34.85 (CH_2, C-aromatic), 41.04 (CH_2, C-aromatic), 120.17 (C, C-aromatic), 121.14 (CH, C-aromatic), 123.00 (CH, C-aromatic), 125.10 (CH, C-aromatic), 126.06 (CH, C-aromatic), 126.74 (CH, C-aromatic), 128.38 (CH, C-aromatic), 128.90 (CH, C-aromatic), 130.39 (CH, C-aromatic), 132.63 (CH, C-aromatic), 132.90 (C, C-aromatic), 135.70 (C, C-aromatic), 137.85 (C, C-aromatic), 139.71 (C, C-aromatic), 163.66 (C, C-aromatic), 168.95 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -62.72 ppm.

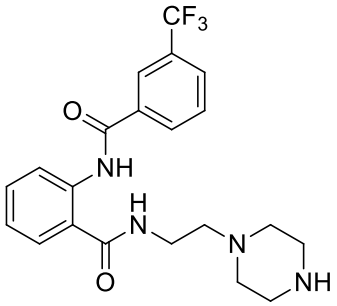
MS (ESI)$^+$: 422.2 $[M+H]^+$

m.p.: (from ethanol/water) 103-105 °C

Synthesis of N-(2-(piperazin-1-yl)ethyl)-2-(3-(trifluoromethyl)benzamido)benzamide (80)

Chemical Formula: $C_{21}H_{23}F_3N_4O_2$

Molecular Weight: 420.43



Procedure: 2

State: white powder

Yield: 36 %

^{1}H-NMR ($CDCl_3$): δ 1.70 (s, 1H), 2.52 (s, 4H), 2.64 (s, 2H), 2.94 (s, 4H), 3.57 (d, *J* = 4.7 Hz, 2H), 7.14 – 7.22 (m, 2H), 7.58 (dd, *J* = 7.4, 13.1 Hz, 2H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.82 (d, *J* = 7.5 Hz, 1H), 8.22 (d, *J* = 7.7 Hz, 1H), 8.38 (s, 1H), 8.84 (d, *J* = 8.1 Hz, 1H), 12.56 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.08 (CH_2, C-aliphatic), 46.12 (CH_2, C-aliphatic), 54.01 (CH_2, C-aliphatic), 56.44 (CH_2, C-aliphatic), 120.22 (C, C-aromatic), 121.61 (CH, C-aromatic), 123.22 (CH, C-aromatic), 125.07 (CH, C-aromatic), 125.10 (CH, C-aromatic), 126.52 (CH, C-aromatic), 128.27 (CH, C-aromatic), 128.30 (CH, C-aromatic), 129.33 (CH, C-aromatic), 130.08(CH, C-aromatic), 131.51 (C, C-aromatic), 132.85 (CH, C-aromatic), 135.88 (C, C-aromatic), 139.99 (C, C-aromatic), 164.04 (C, C-aromatic), 169.00 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 62.90 ppm.

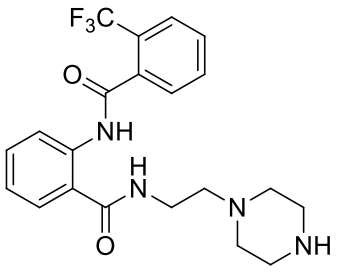
MS (ESI)$^+$: 421.2 $[M+H]^+$

m.p.: (from ethanol/H_2O) 68 – 70 °C

Synthesis of N-(2-(piperazin-1-yl)ethyl)-2-(2-(trifluoromethyl)benzamido)benzamide (81)

Chemical Formula: $C_{21}H_{23}F_3N_4O_2$

Molecular Weight: 420.43



Procedure: 2

State: white powder

Yield: 21 %

^{1}H-NMR ($CDCl_3$): δ 1.76 (s, 1H), 2.50 (s, 4H), 2.59 (dd, *J* = 10.4, 16.3 Hz, 2H), 2.94 (t, *J* = 4.8 Hz, 4H), 3.48 (dd, *J* = 5.7, 10.9 Hz, 2H), 7.03 (s, 1H), 7.20 (td, *J* = 1.1, 7.8 Hz, 1H), 7.52 (dt, *J* = 5.7, 11.3 Hz, 1H), 7.58 (td, *J* = 2.9, 8.1 Hz, 2H), 7.66 (t, *J* = 7.2 Hz, 1H), 7.71 (d, *J* = 7.5 Hz, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 8.80 (d, *J* = 8.3 Hz, 1H), 11.65 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.99 (CH_2, C-aliphatic), 46.05 (CH_2, C-aliphatic), 53.91 (CH_2, C-aliphatic), 56.47 (CH_2, C-aliphatic), 120.46 (C, C-aromatic), 121.76 (CH, C-aromatic), 123.45 (CH, C-aromatic), 126.49 (CH, C-aromatic), 126.73 (CH, C-aromatic), 126.77 (CH, C-aromatic), 128.15 (CH, C-aromatic), 129.99 (CH, C-aromatic), 132.17 (CH, C-aromatic), 132.73 (C, C-aromatic), 136.35 (C, C-aromatic) 139.48 (C, C-aromatic), 166.12 (C, C-aromatic), 168.67 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -58.94 ppm.

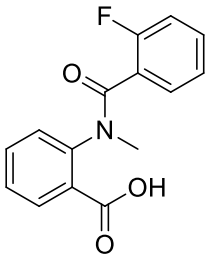
MS (ESI)$^+$: 421.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 116 – 118 °C

Synthesis of 2-(2-fluoro-N-methylbenzamido)benzoic acid (82)

Chemical Formula: $C_{15}H_{12}FNO_3$

Molecular Weight: 273.26



Procedure:

To a solution of *N*-methyl anthranilic acid (1 equivalent) in 30 mL of pyridine, 2-fluorobenzoyl chloride (2 equivalent) was added drop-wise over a period of 30 minutes under an argon atmosphere at 0 °C. The reaction mixture was stirred for 10 hours at room temperature and then poured into ice water (200 mL) and acidified with HCl 37% (25 mL). The precipitate was filtered off and the filtrate was extracted with diethyl ether. The organic phase was dried with $MgSO_4$ and evaporated to dryness to give the titled product.

State: white powder

Yield: 37%

^{1}H-NMR (DMSO-d$_6$): δ 3.51 (s, 3H), 6.20 (s, 1H), 6.81 (t, *J* = 9.0 Hz, 1H), 6.96 (t, *J* = 7.2 Hz, 1H), 7.17 (td, *J* = 1.7, 7.3 Hz, 1H), 7.29 – 7.33 (m, 1H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.50 (td, *J* = 1.5, 7.8 Hz, 1H), 7.92 (dd, *J* = 1.4, 7.8 Hz, 1H), 13.17 (bs, 1H) ppm.

^{13}C-NMR (DMSO-d$_6$): δ 36.91 (CH_3, C-aliphatic), 115.24 (CH, C-aromatic), 123.86 (CH, C-aromatic), 128.05 (CH, C-aromatic), 128.67 (CH, C-aromatic), 128.88 (C, C-aromatic), 129.94 (CH, C-aromatic), 131.00 (CH, C-aromatic), 131.14 (CH, C-aromatic), 132.88 (CH, C-aromatic), 142.58 (C, C-aromatic), 156.00 (C, C-aromatic), 158.16 (C, C-aromatic), 164.79 (C, C-aromatic), 166.27 (C, C-aromatic) ppm.

^{19}F-NMR (DMSO-d$_6$): δ – 112.50 ppm.

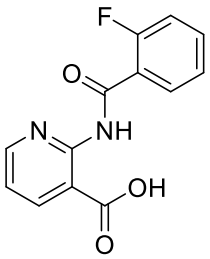
MS(ESI)$^+$: 274.1 $[M+H]^+$

m.p.: (from diethyl ether/*n*-hexane) 128 – 130 °C

Synthesis of 2-(2-fluorobenzamido) nicotinic acid (83)

Chemical Formula: $C_{13}H_9FN_2O_3$

Molecular Weight: 260.22



Procedure: 2

State: white powder

Yield: 27%

^{1}H-NMR (DMSO-d$_6$): δ 7.05 (dd, *J* = 5.0, 7.3 Hz, 1H), 7.28 – 7.36 (m, 2H), 7.57 (ddd, *J* = 1.7, 5.2, 15.1 Hz, 1H), 7.73 (td, *J* = 1.8, 7.6 Hz, 1H), 8.18 – 8.31 (m, 2H), 11.01 (s, 1H), 14.85 (s, 1H) ppm.

^{13}C-NMR (DMSO-d$_6$): δ 118.31 (CH, C-aromatic), 124.54 (CH, C-aromatic), 129.1 (C, C-aromatic), 129.78 (CH, C-aromatic), 132.53 (CH, C-aromatic), 139.30 (CH, C-aromatic), 140. 03 (CH, C-aromatic), 145.46 (C, C-aromatic), 148.13 (CH, C-aromatic), 159.1 (C, C-aromatic), 152.26 (C, C-aromatic), 165.02 (C, C-aromatic), 168.3 (C, C-aromatic) ppm.

^{19}F-NMR (DMSO-d$_6$): δ – 109.57 ppm.

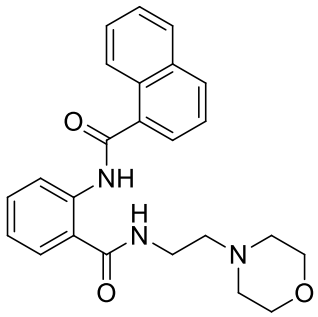
MS(ESI)$^+$: 261.1 $[M+H]^+$

m.p.: > 190 °C

Synthesis of N-(2-((2-morpholinoethyl)carbamoyl)phenyl)-1-naphthamide (84)

Chemical Formula $C_{24}H_{25}N_3O_3$

Molecular Weight: 403.47



Procedure: 2

State: white powder

Yield: 41%

^{1}H-NMR ($CDCl_3$): δ 2.44 (s, 4H), 2.52 (t, *J* = 5.8 Hz, 2H), 3.41 (dd, *J* = 5.5, 1.1 Hz, 2H), 3.59 – 3.72 (m, 4H), 7.11 (td, *J* = 1.1, 7.8 Hz, 1H), 7.44 – 7.58 (m, 6H), 7.80 (dd, *J* = 4.8, 11.9 Hz, 2H), 7.89 (d, *J* = 8.3 Hz, 1H), 8.47 (d, *J* = 8.3 Hz, 1H), 8.84 (d, *J* = 8.0 Hz, 1H), 11.72 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.90 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 56. 54 (CH_2, C-aliphatic), 66.95 (CH_2, C-aliphatic), 120.75 (CH, C-aromatic), 121.76 (C, C-aromatic), 123.12 (CH, C-aromatic), 124.97 (CH, C-aromatic), 125.53 (CH, C-aromatic), 125.67 (CH, C-aromatic), 126.38 (CH, C-aromatic), 126.55 (CH, C-aromatic), 127.12 (CH, C-aromatic), 128.34 (C, C-aromatic), 130.49 (CH, C-aromatic), 131.21 (CH, C-aromatic), 132.70 (C, C-aromatic), 133.95 (CH, C-aromatic), 134.45 (C, C-aromatic), 140.00 (C, C-aromatic), 167.68 (C, C-aromatic), 168.95 (C, C-aromatic) ppm.

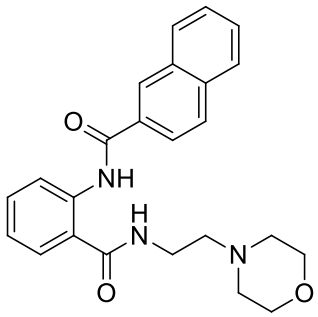
MS (ESI)$^+$: 404.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 93 – 95 °C

Synthesis of N-(2-((2-morpholinoethyl)carbamoyl)phenyl)-2-naphthamide (85)

Chemical Formula $C_{24}H_{25}N_3O_3$

Molecular Weight: 403.47



Procedure: 2

State: white powder

Yield: 48%

^{1}H-NMR ($CDCl_3$): δ 2.57 (s, 4H), 2.68 (t, *J* = 5.8 Hz, 2H), 3.41 – 3.63 (m, 2H), 3.71 – 3.85 (m, 4H), 7.04 (s, 1H), 7.19 (t, *J* = 7.1 Hz, 1H), 7.60 (m, 5H), 7.92 (d, *J* = 7.7 Hz, 1H), 7.99 (d, *J* = 8.6 Hz, 1H), 8.05 (d, *J* = 7.6 Hz, 1H), 8.12 (dd, *J* = 1.7, 8.6 Hz, 1H), 8.61 (s, 1H), 8.90 (d, *J* = 8.2 Hz, 1H), 12.41 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.98 (CH_2, C-aliphatic), 53.30 (CH_2, C-aliphatic), 56.61 (CH_2, C-aliphatic), 66.92 (CH_2, C-aliphatic), 120.47 (C, C-aromatic), 121.79 (CH, C-aromatic), 122.91 (CH, C-aromatic), 123.69 (CH, C-aromatic), 126.53 (CH, C-aromatic), 126.64 (CH, C-aromatic), 127.72 (CH, C-aromatic), 127.79 (CH, C-aromatic), 128.42 (CH, C-aromatic), 128.61 (CH, C-aromatic), 129.39 (CH, C-aromatic), 132.17 (C, C-aromatic), 132.64 (CH, C-aromatic), 132.79 (C, C-aromatic), 134.98 (C, C-aromatic), 140.23 (C, C-aromatic), 165.52 (C, C-aromatic), 169.19 (C, C-aromatic) ppm.

MS (ESI)$^+$: 404.2 $[M+H]^+$

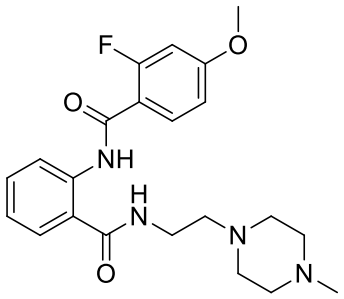
m.p.: (from dichloromethane/*n*-hexane) 105 – 107 °C

HPLC (method 1): retention time 26.6 minutes.

Synthesis of 2-fluoro-4-methoxy-N-(2-((2-(4-methylpiperazin-1- yl)ethyl)carbamoyl)phenyl) benzamide (86)

Chemical Formula: $C_{22}H_{27}FN_4O_3$

Molecular Weight: 414.47



Procedure: 2

State: white powder

Yield: 23%

^{1}H-NMR ($CDCl_3$): δ 2.29 (s, 3H), 2.41 – 2.66 (m, 10H), 3.48 (dd, *J* = 11.2, 5.4 Hz, 2H), 3.79 (s, 3H), 6.62 (dd, *J* = 2.4, 13.3 Hz, 1H), 6.73 (dd, *J* = 2.4, 8.8 Hz, 1H), 6.84 (s, 1H), 7.07 (td, *J* = 1.1, 7.6 Hz, 1H), 7.44 (t, *J* = 7.3 Hz, 2H), 7.96 (t, *J* = 8.9 Hz, 1H), 8.64 (d, *J* = 7.9 Hz, 1H), 11.59 (d, *J* = 7.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.18 (CH_2, C-aliphatic), 45.61 (CH_2, C-aliphatic), 52.31 (CH_2, C-aliphatic), 54.80 (CH_2, C-aliphatic), 55.81 (CH_3, C-aliphatic), 56.08 (CH_2, C-aliphatic), 101.77 (CH, C-aromatic), 101.98 (CH, C-aromatic), 110.69 (C, C-aromatic), 110.71 (CH, C-aromatic), 122.04 (CH, C-aromatic), 122.62 (CH, C-aromatic), 123.16 (CH, C-aromatic), 126.64 (C, C-aromatic), 132.90 (CH, C-aromatic), 139.22 (C, C-aromatic), 160.52 (C, C-aromatic), 162.39 (C, C-aromatic), 163.65 (C, C-aromatic), 168.58 (C, C-aromatic) ppm.

MS (ESI)$^+$: 415.2 $[M+H]^+$

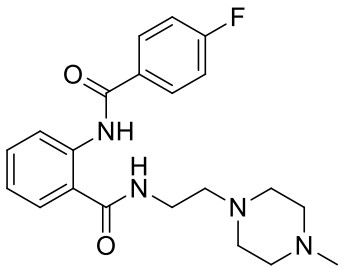
m.p.: (from dichloromethane/*n*-hexane) 110 - 112 °C

HPLC (method 1): retention time 11.24 minutes

Synthesis of 2-(4-fluorobenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (87)

Chemical Formula: $C_{21}H_{25}FN_4O_2$

Molecular Weight: 384.45



Procedure: 2

State: yellow powder

Yield: 44%

^{1}H-NMR ($CDCl_3$): δ 2.24 (d, *J* = 9.6 Hz, 3H), 2.50 (s, 7H), 2.54 – 2.61 (m, 3H), 3.47 (dd, *J* = 5.6, 10.9 Hz, 2H), 7.00 (s, 1H), 7.08 – 7.16 (m, 3H), 7.39 - 7.51 (m, 2H),8.03 – 7.93 (m, 2H), 8.79 – 8.68 (m, 1H), 12.26 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.24 (CH_2, C-aliphatic), 45.96 (CH_3, C-aliphatic), 52.67 (CH_2, C-aliphatic), 55.13 (CH_2, C-aliphatic), 55.80 (CH_2, C-aliphatic), 115.80 (CH, C-aromatic), 120.16 (C, C-aromatic), 121.53 (CH, C-aromatic), 122.93 (CH, C-aromatic), 126.56 (CH, C-aromatic), 129.79 (CH, C-aromatic), 129.87 (CH, C-aromatic), 132.81 (C, C-aromatic), 140.17 (C, C-aromatic), 163.70 (C, C-aromatic), 164.46 (C, C-aromatic), 166.10 (C, C-aromatic), 169.12 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -107.64 ppm.

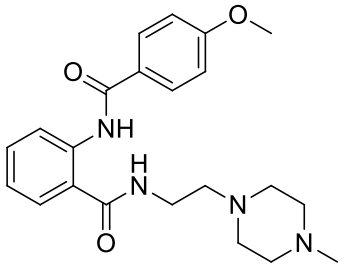
MS (ESI)$^{+}$: 385.2 $[M+H]^{+}$

m.p.: (from diethyl ether/*n*-hexane) 89 - 91 °C

Synthesis of 2-(4-methoxybenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (88)

Chemical Formula: $C_{22}H_{28}N_4O_3$

Molecular Weight: 396.48



Procedure: 2

State: white powder

Yield: 24%

^{1}H-NMR ($CDCl_3$): δ 2.22 (s, 3H), 2.34 – 2.51 (m, 7H), 2.51 – 2.58 (m, 3H), 3.45 (dd, *J* = 5.7, 11.1 Hz, 2H), 3.77 (s, 3H), 6.91 (d, *J* = 8.8 Hz, 2H), 6.97 – 7.02 (m, 1H), 7.05 (s, 1H), 7.42 (t, *J* = 7.9 Hz, 2H), 7.92 (d, *J* = 8.8 Hz, 2H), 8.71 (d, *J* = 8.2 Hz, 1H), 12.06 (d, *J* = 17.3 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.35 (CH_2, C-aliphatic), 45.87 (CH_3, C-aliphatic), 52.63 (CH_2, C-aliphatic), 55.06 (CH_2, C-aliphatic), 55.41 (CH_3, C-aliphatic), 56.60 (CH_2, C-aliphatic), 113.96 (CH, C-aromatic), 120.27 (C, C-aromatic), 122.55 (CH, C-aromatic), 125.49 (CH, C-aromatic), 126.85 (CH, C-aromatic), 127.24 (C, C-aromatic), 129.12 (CH, C-aromatic), 132.54 (CH, C-aromatic), 140.29 (C, C-aromatic), 162.49 (C, C-aromatic), 165.14 (C, C-aromatic), 169.19 (C, C-aromatic) ppm.

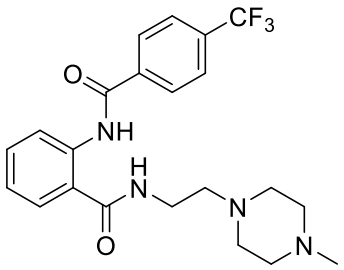
MS (ESI)$^+$: 397.2 $[M+H]^+$

m.p.: (from diethyl ether/*n*-hexane) 88-90 °C

Synthesis of N-(2-(4-methylpiperazin-1-yl)ethyl)-2-(4-(trifluoromethyl)benzamido)benzamide (89)

Chemical Formula: $C_{22}H_{25}F_3N_4O_2$

Molecular Weight: 434.46



Procedure: 2

State: yellow powder

Yield: 23%

^{1}H-NMR ($CDCl_3$): δ 2.26 (s, 3H), 2.49 (d, J = 41.4 Hz, 7H), 2.57 – 2.62 (m, 2H), 3.45 – 3.50 (m, 2H), 7.04 – 7.13 (m, 2H), 7.46 – 7.53 (m, 2H), 7.70 (d, J = 8.2 Hz, 2H), 8.09 (d, J = 8.1 Hz, 2H), 8.76 (d, J = 7.8 Hz, 1H), 12.46 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.23 (CH_2, C-aliphatic), 45.82 (CH_3, C-aliphatic), 52.56 (CH_2, C-aliphatic), 54.97 (CH_2, C-aliphatic), 55.89 (CH_2, C-aliphatic), 120.12 (C, C-aromatic), 121.57 (CH, C-aromatic), 123.26 (CH, C-aromatic), 125.76 (CH, C-aromatic), 125.82 (CH, C-aromatic), 126.61 (CH, C-aromatic), 127.87 (CH, C-aromatic), 132.89 (C, C-aromatic), 133.26 (C, C-aromatic), 138.26 (C, C-aromatic), 139.99 (C, C-aromatic), 164.17 (C, C-aromatic), 169.06 (C, C-aromatic) ppm.

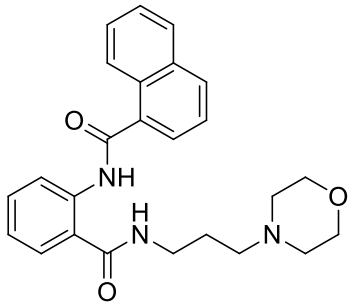
^{19}F-NMR ($CDCl_3$): δ - 63.1 ppm.

MS (ESI)$^+$: 435.2 $[M+H]^+$

m.p.: (from diethyl ether/*n*-hexane) 68 - 70 °C

Synthesis N-(2-((3-morpholinopropyl)carbamoyl)phenyl)-1-naphthamide (90)

Molecular formula: $C_{25}H_{27}N_3O_3$.
Molecular weight: 417.19



Procedure: 2

State: white powder

Yield: 74 %

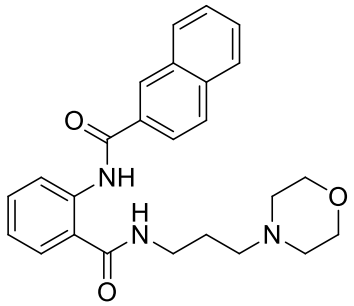
^{1}H-NMR ($CDCl_3$): δ 1.77 (2H, m), 2.56 (3H, s), 2.58 (3H, s), 3.52 (2H, dd, *J* = 5.7, 10.9 Hz), 3.74 (4H, s), 7.20 (1H, td, *J* = 1.05, 7.62 Hz), 7.55 - 7.75 (5H, m), 7.91 (2H, m), 7.98 (1H, d, *J* = 8.33 Hz), 8.57 (2H, m), 8.95 (1H, m), 12.03 (1H, s) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.38 (CH_2, C-aliphatic), 40.62 (CH_2, C-aliphatic), 53.72 (CH_2, C-aliphatic), 58.68 (CH_2, C-aliphatic), 66.65 (CH_2, C-aliphatic), 120.72 (C, C-aromatic), 121.64 (CH, C-aromatic), 122.68 (CH, C-aromatic),125.00 (CH, C-aromatic), 125.56 (CH, C-aromatic), 125.70 (CH, C-aromatic), 126.35 (CH, C-aromatic), 126.88 (CH, C-aromatic), 127.08 (CH, C-aromatic), 128.32 (CH, C-aromatic), 130.52 (C, C-aromatic), 131.15 (CH, C-aromatic), 132.63 (CH, C-aromatic), 133.95 (C, C-aromatic), 134.51 (C, C-aromatic), 140.08 (C, C-aromatic), 167.90 (C, C-aromatic), 168.91 (C, C-aromatic) ppm.
MS (ESI)$^+$: 418.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 110 – 112 °C

Synthesis N-(2-((3-morpholinopropyl)carbamoyl)phenyl)-2-naphthamide (91)

Molecular formula: $C_{25}H_{27}N_3O_3$.
Molecular weight: 417.19



Procedure: 2

State: white powder

Yield: 53%

^{1}H-NMR ($CDCl_3$): δ 1.85 (2H, m), 2.56 (4H, m), 2.64 (2H, m), 3.63 (2H, m), 3.74 (4H, m), 7.18 (1H, dd), 7.59 (3H, m), 7.65 (1H, m), 7.92 (1H, d, *J* = 7.79 Hz), 7.99 (1H, s), 8.05 (1H, m), 8.14 (1H, m), 8.63 (2H, m), 8.90 (1H, d, *J* = 8.43 Hz), 12.58 (1H, s) ppm.

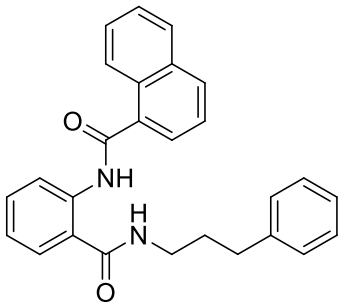
^{13}C-NMR ($CDCl_3$): δ 23.44 (CH_2, C-aliphatic), 40.91 (CH_2, C-aliphatic), 53.80 (CH_2, C-aliphatic), 58.88 (CH_2, C-aliphatic), 66.76 (CH_2, C-aliphatic), 120.57 (C, C-aromatic), 121.70 (CH, C-aromatic), 122.64 (CH, C-aromatic), 123.72 (CH, C-aromatic), 126.60 (CH, C-aromatic), 126.83 (CH, C-aromatic), 127.70 (CH, C-aromatic), 128.43 (CH, C-aromatic), 128.59 (CH, C-aromatic), 129.40 (CH, C-aromatic), 132.23 (C, C-aromatic), 132.66 (CH, C-aromatic), 132.82 (C, C-aromatic), 134.96 (C, C-aromatic), 140.24 (C, C-aromatic), 165.69 (C, C-aromatic), 169.22 (C, C-aromatic) ppm.

MS (ESI)$^+$: 418.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 103-105 °C

Synthesis N-(2-((3-phenylpropyl)carbamoyl)phenyl)-1-naphthamide (92)

Molecular formula: $C_{27}H_{24}N_2O_2$.
Molecular weight: 408.49



Procedure: 2

State: white powder

Yield: 59%

^{1}H-NMR ($CDCl_3$): δ 1.97 (2H, q, *J* = 7.22 Hz), 2.74 (2H, t, *J* = 7.40 Hz), 3.46 (2H, m), 6.16 (1H, s), 7.13 (1H, dd, *J* = 1.16, 7.43 Hz), 7.19 - 7.25 (4H, m), 7.30 (2H, dd, *J* = 4.4, 12.3 Hz), 7.57 (4H, m), 7.90 (2H, m), 7.99 (1H, d, *J* = 8.29 Hz), 8.56 (1H, m), 8.91 (1H, dd, *J* = 0.90, 8.45 Hz), 11.71 (1H, s) ppm.

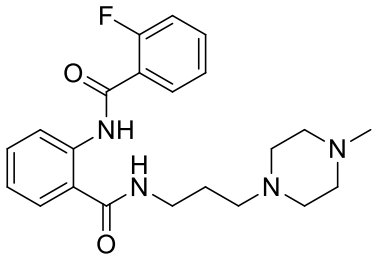
^{13}C-NMR ($CDCl_3$): δ 30.80 (CH_2, C-aliphatic), 33.54 (CH_2, C-aliphatic), 39.80 (CH_2, C-aliphatic), 120.96 (C, C-aromatic), 121.71 (CH, C-aromatic), 123.04 (CH, C-aromatic), 124.99 (CH, C-aromatic), 125.56 (CH, C-aromatic), 125.63 (CH, C-aromatic), 126.18 (CH, C-aromatic), 126.38 (CH, C-aromatic), 126.43 (CH, C-aromatic), 127.13 (CH, C-aromatic), 128.36 (CH, C-aromatic), 128.65 (CH, C-aromatic), 130.46 (C, C-aromatic), 131.22 (CH, C-aromatic), 132.57 (CH, C-aromatic), 133.94 (C, C-aromatic), 134.40 (C, C-aromatic), 139.72 (C, C-aromatic), 141.22 (C, C-aromatic), 167.93 (C, C-aromatic), 168.82 (C, C-aromatic) ppm.

MS (ESI)$^+$: 431.2 $[M+Na]^+$

m.p.: (from ethanol/water) 98 - 100 °C

Synthesis 2-fluoro-N-(2-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)benzamide (93)

Molecular formula: $C_{22}H_{27}FN_4O_2$.
Molecular weight: 398.48



Procedure: 2

State: white powder

Yield: 56 %

^{1}H-NMR ($CDCl_3$): δ 1.19 (3H, m), 1.72 (2H, dd, *J* = 4.9, 10.2 Hz), 2.22 (3H, s), 2.29 – 2.57 (7H, m), 3.49 (2H, m), 7.05 – 7.14 (3H, m), 7.18 – 7.23 (1H, m), 7.39 – 7.48 (1H, m), 7.54 (1H, d, *J* = 6.8 Hz), 7.97 (1H, td, *J* = 1.8, 7.7 Hz), 8.71 (1H, d, *J* = 8.4 Hz), 8.79 (1H, d, *J* = 6.1 Hz), 12.03 (1H, s) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.57 (CH_2, C-aliphatic), 41.21 (CH_2, C-aliphatic), 46.01 (CH_3, C-aliphatic), 53.21 (CH_2, C-aliphatic), 54.97 (CH_2, C-aliphatic), 58.56 (CH_2, C-aliphatic), 116.55 (CH, C-aromatic), 121.78 (C, C-aromatic), 122.25 (CH, C-aromatic), 122.99 (CH, C-aromatic), 124.55 (CH, C-aromatic), 127.33 (CH, C-aromatic), 131.42 (CH, C-aromatic), 132.20 (CH, C-aromatic), 133.19 (CH, C-aromatic), 139.31 (C, C-aromatic), 159.37 (C, C-aromatic), 161.38 (C, C-aromatic), 162.42 (C, C-aromatic), 168.64 (C, C-aromatic) ppm.

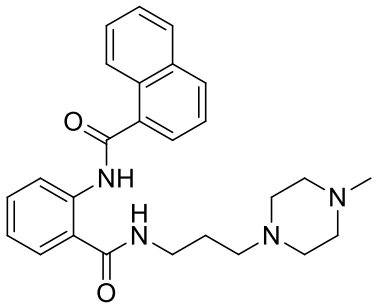
^{19}F-NMR ($CDCl_3$): δ -112.45 ppm.

MS(ESI)$^+$: 399.2 $[M+Na]^+$

m.p.: 69 - 71 °C

Synthesis N-(2-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)-1-naphthamidebenzamide (94)

Molecular formula: $C_{26}H_{30}N_4O_2$.
Molecular weight: 430.55



Procedure: 2

State: white powder

Yield: 69 %

^{1}H-NMR ($CDCl_3$): δ 1.68 (2H, m), 2.23 (3H, s), 2.31 – 2. 49 (8H, m), 2.51 (2H, d, *J* = 5.59 Hz), 3.43 (2H, m), 7.07 (1H, m), 7.51 (5H, m), 7.81 (2H, ddd, *J* = 7.61, 3.12, 1.17 Hz), 7.88 (1H, d, *J* = 0.31 Hz), 8.50 (1H, m), 8.84 (2H, m), 12.02 (1H, t, *J* = 0.37 Hz) ppm.

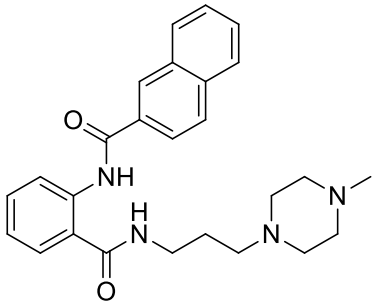
^{13}C-NMR ($CDCl_3$): δ 23.43 (CH_2, C-aliphatic), 41.37 (CH_2, C-aliphatic), 46.10 (CH_3, C-aliphatic), 53.38 (CH_2, C-aliphatic), 55.10 (CH_2, C-aliphatic), 58.75 (CH_2, C-aliphatic), 120.75 (C, C-aromatic), 121.51 (CH, C-aromatic), 122.71 (CH, C-aromatic), 125.02 (CH, C-aromatic), 125.58 (CH, C-aromatic), 125.72 (CH, C-aromatic), 126.32 (CH, C-aromatic), 127.05 (CH, C-aromatic), 127.39 (CH, C-aromatic), 128.31 (CH, C-aromatic), 130.53 (C, C-aromatic), 131.12 (CH, C-aromatic), 132.49 (CH, C-aromatic), 133.96 (C, C-aromatic), 134.54 (C, C-aromatic), 140.13 (C, C-aromatic), 167.94 (C, C-aromatic), 168.82 (C, C-aromatic) ppm.

MS (ESI)$^+$: 431.2 $[M+H]^+$

m.p.: 98 - 100 °C

Synthesis N-(2-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)-2-naphthamide (95)

Molecular formula: $C_{26}H_{30}N_4O_2$.
Molecular weight: 430.55



Procedure: 2

State: white powder

Yield: 95 %

^{1}H-NMR ($CDCl_3$): δ 1.30 (m, 3H), 1.84 (m, 2H), 2.33 (s, 3H), 2.38 – 2.57 (m, 5H), 2.65 (d, 2H, *J* = 5.47 Hz), 3.36 (m, 2H), 7.16 (t, *J* = 0.95, 7.59 Hz, 1H), 7.29 (1H, s), 7.60 (dd, *J* = 6.42 Hz, 3H), 7.69 (dd, *J* = 1.30, 7.87 Hz, 1H), 7.93 (d, *J* = 0.39 Hz, 1H), 7.99 (s, 1H), 8.06 (m, 1H), 8.14 (d, *J* = 6.74 Hz, 1H), 8.63 (t, *J* = 0.62 Hz, 1H), 8.95 (m, 2H), 12.67 (s, 1H) ppm.

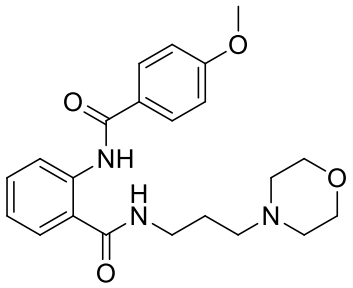
^{13}C-NMR ($CDCl_3$): δ 23.50 (CH_2, C-aliphatic), 41.32 (CH_2, C-aliphatic), 46.02 (CH_3, C-aliphatic), 53.29 (CH_2, C-aliphatic), 54.98 (CH_2, C-aliphatic), 58.62 (CH_2, C-aliphatic), 120.56 (C, C-aromatic), 121.58 (CH, C-aromatic), 122.55 (CH, C-aromatic), 123.75 (CH, C-aromatic), 126.58 (CH, C-aromatic), 127.37 (CH, C-aromatic), 127.70 (CH, C-aromatic), 127.73 (CH, C-aromatic), 128.44 (CH C-aromatic), 128.59 (CH, C-aromatic), 129.42 (CH, C-aromatic), 132.26 (C, C-aromatic), 132.55 (CH, C-aromatic), 132.82 (C, C-aromatic), 134.96 (C, C-aromatic), 140.26 (C, C-aromatic), 165.00 (C, C-aromatic), 169.18 (C, C-aromatic) ppm.

MS (ESI)$^+$: 431.2 $[M+H]^+$

m.p.: 70 - 72 °C

Synthesis 2-(4-methoxybenzamido)-N-(3-morpholinopropyl)benzamide (96)

Molecular formula: $C_{22}H_{27}N_3O_4$
Molecular weight: 397.47



Procedure: 2

State: white powder

Yield: 64 %

^{1}H-NMR ($CDCl_3$): δ 1.85 (2H, m), 2.54 (4H, m), 2.63 (2H, dd, *J* = 3.88, 7.36 Hz), 3.61 (2H, m), 3.74 (4H, t, *J* = 4.29 Hz), 3.90 (3H, s), 7.02 (2H, m), 7.13 (1H, td, *J* = 0.98, 7.59 Hz), 7.55 (1H, m), 7.62 (1H, dd, *J* = 1.03, 7.80 Hz), 8.05 (2H, m), 8.60 (1H, m), 8.84 (1H, dd, *J* = 1.00, 8.44 Hz), 12.32 (1H, s) ppm.

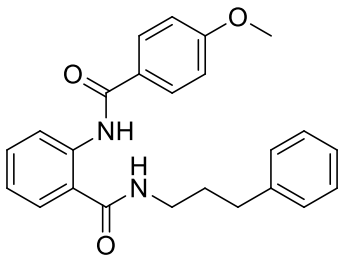
^{13}C-NMR ($CDCl_3$): δ 23.41 (CH_2, C-aliphatic), 40.97 (CH_2, C-aliphatic), 53.82 (CH_2, C-aliphatic), 55.42 (CH_3, C-aliphatic), 58.95 (CH_2, C-aliphatic), 66.79 (CH_2, C-aliphatic), 113.95 (CH, C-aromatic), 120.23 (C, C-aromatic), 121.52 (CH, C-aromatic), 122.30 (CH, C-aromatic), 126.77 (CH, C-aromatic), 127.34 (C, C-aromatic), 129.33 (CH, C-aromatic), 132.62 (CH, C-aromatic), 140.41 (C, C-aromatic), 162.46 (C, C-aromatic), 165.19 (C, C-aromatic), 169.28 (C, C-aromatic) ppm.

MS (ESI)$^+$: 398.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 112 - 114 °C

Synthesis 2-(4-methoxybenzamido)-N-(3-phenylpropyl)benzamide (97)

Chemical Formula: $C_{24}H_{24}N_2O_3$
Molecular Weight: 388.46



Procedure: 2

State: white powder

Yield: 62%

^{1}H-NMR ($CDCl_3$): δ 2.03 (dd, *J* = 7.2, 14.4 Hz, 2H), 2.79 (t, *J* = 7.4 Hz, 2H), 3.49 – 3.56 (m, 2H), 3.90 (s, 3H), 6.25 (s, 1H), 6.99 – 7.07 (m, 3H), 7.20 – 7.28 (m, 3H), 7.29 – 7.36 (m, 3H), 7.49 – 7.55 (m, 1H), 8.01 – 8.05 (m, 2H), 8.75 – 8.83 (m, 1H), 12.04 (s, 1H) ppm.

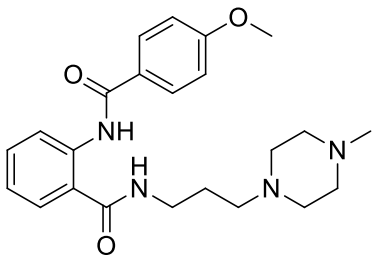
^{13}C-NMR ($CDCl_3$): δ 30.82 (CH_2, C-aliphatic), 33.59 (CH_2, C-aliphatic), 39.87 (CH_2, C-aliphatic), 55.3 (CH_3, C-aliphatic), 113.98 (CH, C-aromatic), 120.42 (C, C-aromatic), 121.53 (CH, C-aromatic), 122.47 (CH, C-aromatic), 126.20 (CH, C-aromatic), 126.35 (CH, C-aromatic), 127.24 (C, C-aromatic), 128.39 (CH, C-aromatic), 128.67 (CH, C-aromatic), 129.31 (CH, C-aromatic), 132.57 (CH, C-aromatic), 140.11 (C, C-aromatic), 141.25 (C, C-aromatic), 162.51 (C, C-aromatic), 165.18 (C, C-aromatic), 169.21 (C, C-aromatic) ppm.

MS (ESI)$^+$: 389.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 92 – 94 °C

Synthesis of 2-(4-methoxybenzamido)-N-(3-(4-methylpiperazin-1-yl)propyl)benzamide (98)

Chemical Formula: $C_{23}H_{30}N_4O_3$
Molecular Weight: 410.51



Procedure: 2

State: white powder

Yield: 69%

^{1}H-NMR ($CDCl_3$): δ 1.84 (s, 2H), 2.33 (s, 3H), 2.53 (s, 6H), 2.65 (s, 4H), 3.61 (d, *J* = 4.9 Hz, 2H), 3.90 (s, 3H), 7.02 (d, *J* = 8.7 Hz, 2H), 7.12 (t, *J* = 7.5 Hz, 1H), 7.55 (t, *J* = 7.6 Hz, 1H), 7.66 (d, *J* = 7.7 Hz, 1H), 8.05 (d, *J* = 8.7 Hz, 2H), 8.85 (d, *J* = 8.4 Hz, 1H), 8.96 (s, 1H), 12.42 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.41 (CH_2, C-aliphatic), 41.25 (CH_2, C-aliphatic), 46.01 (CH_3, C-aliphatic), 53.22 (CH_2, C-aliphatic), 54.88 (CH_2, C-aliphatic), 55.44 (CH_3, C-aliphatic), 58.44 (CH_2, C-aliphatic), 113.94 (CH, C-aromatic), 120.24 (C, C-aromatic), 121.36 (CH, C-aromatic), 122.25 (CH, C-aromatic), 127.32 (C, C-aromatic), 127.37 (CH, C-aromatic), 129.34 (CH, C-aromatic), 132.55 (CH, C-aromatic), 140.42 (C, C-aromatic), 162.43 (C, C-aromatic), 165.22 (C, C-aromatic), 169.25 (C, C-aromatic) ppm.

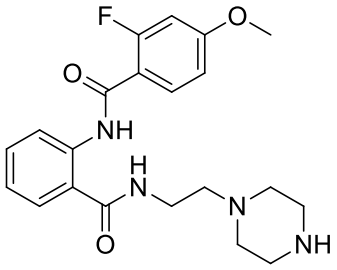
MS (ESI)$^+$: 411.2 $[M+H]^+$

m.p.: (from acetone/*n*-hexane) 79 – 81 °C

Synthesis of 2-fluoro-4-methoxy-N-(2-((2-(piperazin-1-yl)ethyl)carbamoyl)phenyl)benzamide (99)

Chemical Formula: $C_{21}H_{25}FN_4O_3$

Molecular Weight: 400.45



Procedure: 2

State: White powder

Yield: 33%

^{1}H-NMR ($CDCl_3$): δ 1.19 (s, 1H), 2.48 (s, 3H), 2.52 – 2.57 (m, 2H), 2.86 – 2.95 (m, 4H), 3.46 (dd, J = 5.5, 11.1 Hz, 2H), 3.79 (s, 3H), 6.62 (dd, J = 2.4, 13.3 Hz, 1H), 6.72 (d, J = 2.4 Hz, 1H), 6.78 (s, 1H), 7.03 – 7.09 (m, 1H), 7.38 – 7.51 (m, 2H), 7.97 (dd, J = 7.9, 16.8 Hz, 1H), 8.64 (d, J = 8.4 Hz, 1H), 11.59 (d, J = 8.4 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.11 (CH_2, C-aliphatic), 45.18 (CH_2, C-aliphatic), 52.49 (CH_2, C-aliphatic), 55.82 (CH_3, C-aliphatic), 56.55 (CH_2, C-aliphatic), 101.78 (CH, C-aromatic), 101.99 (CH, C-aromatic), 110.70 (CH, C-aromatic), 122.11 (CH, C-aromatic), 122.65 (C, C-aromatic), 123.21 (CH, C-aromatic), 126.61 (CH, C-aromatic), 132.27 (CH, C-aromatic), 139.17 (C, C-aromatic), 160.74 (C, C-aromatic), 161.86 (C, C-aromatic), 162.40 (C, C-aromatic), 163.57 (C, C-aromatic), 168.73 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.44 ppm.

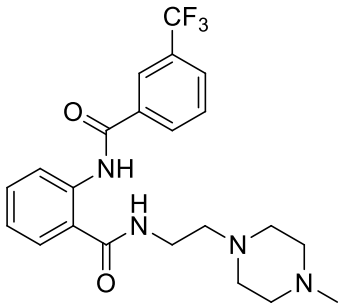
MS (ESI)$^{+}$: 401.2 $[M+H]^{+}$

m.p.: (from dichloromethane/*n*-hexane) 107 – 109 °C

Synthesis of N-(2-(4-methylpiperazin-1-yl)ethyl)-2-(3-(trifluoromethyl)benzamido)benzamide (100)

Chemical Formula: $C_{22}H_{25}F_3N_4O_2$

Molecular Weight: 434.45



Procedure: 2

State: white powder

Yield: 40 %

^{1}H-NMR ($CDCl_3$): δ 2.27 (s, 3H), 2.64 – 2.39 (m, 10H), 3.49 (dd, *J* = 5.3, 11.2 Hz, 2H), 7.10 (dd, *J* = 4.1, 11.0 Hz, 2H), 7.46 – 7.52 (m, 2H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.73 (d, *J* = 7.8 Hz, 1H), 8.13 (d, *J* = 7.8 Hz, 1H), 8.29 (s, 1H), 8.74 (d, *J* = 8.3 Hz, 1H), 12.46 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.19 (CH_2, C-aliphatic), 45.82 (CH_3, C-aliphatic), 52.53 (CH_2, C-aliphatic), 54.93 (CH_2, C-aliphatic), 5.89 (CH_2, C-aliphatic), 120.14 (CH, C-aromatic), 121.59 (CH, C-aromatic), 123.26 (CH, C-aromatic), 125.10 (CH, C-aromatic), 126.63 (CH, C-aromatic), 128.34 (CH, C-aromatic), 129.37 (CH, C-aromatic), 130.07 (C, C-aromatic), 132.90 (CH, C-aromatic), 135.82 (C, C-aromatic), 139.93 (C, C-aromatic), 164.06 (C, C-aromatic), 169.07 (C, C-aromatic), 171.98 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -62.69 ppm.

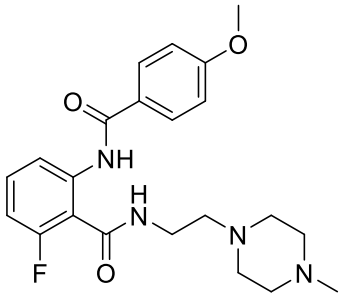
MS (ESI)$^+$: 435.2 $[M+H]^+$

m.p.: 101 – 103 °C

Synthesis of 2-fluoro-6-(4-methoxybenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (101)

Chemical Formula: $C_{22}H_{27}FN_4O_3$

Molecular Weight: 414.47



Procedure: 2

State: white powder

Yield: 45 %

^{1}H-NMR ($CDCl_3$): δ 2.36 (s, 3H), 2.48 -2.81 (m, 10H), 3.41 – 3.60 (m, 2H), 3.81 (s, 3H), 6.79 (dd, *J* = 8.3, 11.6 Hz, 1H), 6.93 (d, *J* = 8.8 Hz, 2H), 7.40 (dt, *J* = 10.2, 15.0 Hz, 2H), 7.94 (d, *J* = 8.9 Hz, 2H), 8.58 (d, *J* = 8.5 Hz, 1H), 12.40 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.48 (CH_2, C-aliphatic), 45.16 (CH_3, C-aliphatic), 51.46 (CH_2, C-aliphatic), 54.68 (CH_2, C-aliphatic), 55.38 (CH_2, C-aliphatic), 55.48 (CH_3, C-aliphatic), 108.49 (C, C-aromatic), 109.89 (CH, C-aromatic), 114.01 (CH, C-aromatic), 117.44 (CH, C-aromatic), 128.88 (CH, C-aromatic), 132.96 (C, C-aromatic), 142.18 (CH, C-aromatic), 142.22 (C, C-aromatic), 159.95 (C, C-aromatic), 161.91 (C, C-aromatic), 162.63 (C, C-aromatic), 165.50 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.87 ppm.

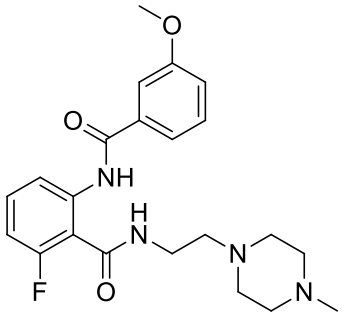
MS (ESI)$^+$: 415.2 $[M+H]^+$

m.p.: (from diethyl ether/*n*-hexane) 81 - 83°C

Synthesis of 2-fluoro-6-(3-methoxybenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (102)

Chemical Formula: $C_{22}H_{27}FN_4O_3$

Molecular Weight: 414.47



Procedure: 2

State: white powder

Yield: 28 %

^{1}H-NMR ($CDCl_3$): δ 2.30 (s, 3H), 2.41 -2.73 (m, 10H), 2.80 (s, 3H), 3.48 – 3.55 (m, 2H), 6.79 (dd, J = 8.3, 11.6 Hz, 1H), 6.93 (d, J = 8.8 Hz, 2H), 7.40 (dt, J = 10.2, 15.0 Hz, 2H), 7.94 (d, J = 8.9 Hz, 2H), 8.58 (d, J = 8.5 Hz, 1H), 12.20 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.43 (CH_2, C-aliphatic), 51.76 (CH_2, C-aliphatic), 55.39 (CH_2, C-aliphatic), 55.47 (CH_3, C-aliphatic), 55.62 (CH_2, C-aliphatic), 112.56 (CH, C-aromatic), 119.23 (CH, C-aromatic), 121.06 (CH, C-aromatic), 123.14 (CH, C-aromatic), 123.18 (CH, C-aromatic), 129.88 (CH, C-aromatic), 131.04 (C, C-aromatic), 137.28 (CH, C-aromatic), 137.36 (C, C-aromatic), 142.22 (C, C-aromatic)148.74 (C, C-aromatic), 159.90 (C, C-aromatic), 160.86 (C, C-aromatic), 162.99 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -106.45 ppm.

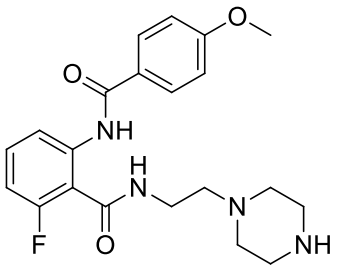
MS (ESI)$^{+}$: 415.2 $[M+H]^{+}$

m.p.: (from ethanol/water) 108 - 110°C

Synthesis of 2-fluoro-6-(4-methoxybenzamido)-N-(2-(piperazin-1-yl)ethyl)benzamide (103)

Chemical Formula: $C_{21}H_{25}FN_4O_3$

Molecular Weight: 400.45



Procedure: 2

State: white powder

Yield: 34 %

^{1}H-NMR ($CDCl_3$): δ 2.25 (d, *J* = 14.7 Hz, 4H), 2.58 (dd, *J* = 15.7, 21.6 Hz, 8H), 3.49 (s, 3H), 7.05 (s, 1H), 7.09 (dd, *J* = 9.5, 16.9 Hz, 1H), 7.44 – 7.52 (m, 2H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.73 (d, *J* = 7.8 Hz, 1H), 8.13 (d, *J* = 7.8 Hz, 1H), 8.29 (s, 1H), 8.75 (d, *J* = 8.4 Hz, 1H), 12.46 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.37 (CH_2, C-aliphatic), 46.13 (CH_2, C-aliphatic), 53.98 (CH_2, C-aliphatic), 55.47 (CH_3, C-aliphatic), 56.03 (CH_2, C-aliphatic), 109.92 (CH, C-aromatic), 113.99(CH, C-aromatic), 117.33 (CH, C-aromatic), 127.07 (C, C-aromatic), 129.40 (CH, C-aromatic), 132.89 (CH, C-aromatic), 132.98, 141.87 (C, C-aromatic), 159.62 (C, C-aromatic), 161.79 (C, C-aromatic), 162.57 (C, C-aromatic), 165.27 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.78 ppm.

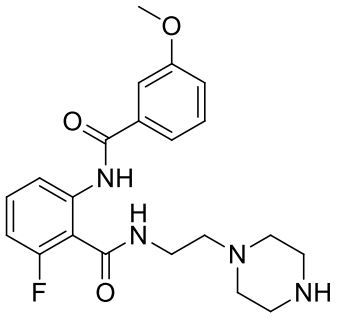
MS(ESI)$^+$: 401.2 $[M+H]^+$

m.p.: (from ethanol/water) 96 - 98°C

Synthesis of 2-fluoro-6-(3-methoxybenzamido)-N-(2-(piperazin-1-yl)ethyl)benzamide (104)

Chemical Formula: $C_{21}H_{25}FN_4O_3$

Molecular Weight: 400.45



Procedure: 2

State: white powder

Yield: 45 %

^{1}H-NMR ($CDCl_3$): δ 1.30 (s, 1H), 2.04 (d, *J* = 32.0 Hz, 1H), 2.52 (s, 3H), 2.61 (dd, *J* = 10.8, 16.8 Hz, 2H), 2.95 (t, *J* = 4.4 Hz, 4H), 3.59 (d, *J* = 5.2 Hz, 2H), 3.91 (s, 3H), 6.90 (dd, *J* = 8.5, 11.7 Hz, 1H), 7.09 – 7.14 (m, 1H), 7.41 – 7.52 (m, 2H), 7.60 – 7.70 (m, 3H), 8.70 (d, *J* = 8.4 Hz, 1H), 12.67 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.35 (CH_2, C-aliphatic), 45.85 (CH_2, C-aliphatic), 53.48 (CH_2, C-aliphatic), 55.46 (CH_3, C-aliphatic), 55.97 (CH_2, C-aliphatic), 108.99 (C, C-aromatic), 110.26 (CH, C-aromatic), 110.46 (CH, C-aromatic), 112.50 (CH, C-aromatic), 117.42 (CH, C-aromatic), 118.51 (CH, C-aromatic), 119.26 (CH, C-aromatic), 129.84 (CH, C-aromatic), 132.97 (C, C-aromatic), 136.24 (C, C-aromatic), 141.88 (C, C-aromatic), 159.94 (C, C-aromatic), 161.79 (C, C-aromatic), 165.55 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.66 ppm.

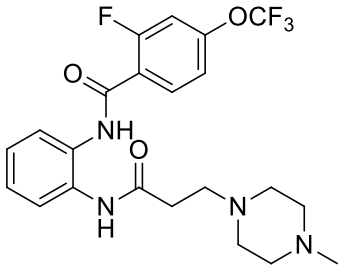
MS (ESI)$^+$: 401.2 $[M+H]^+$

m.p.: (from ethanol/water) 83 – 85 °C

Synthesis of 2-fluoro-N-(2-(3-(4-methylpiperazin-1-yl)propanamido)phenyl)-4-(trifluoromethoxy)benzamide (105)

Chemical Formula: $C_{22}H_{24}F_4N_4O_3$

Molecular Weight: 468.44



Procedure: 2

State: white powder

Yield: 34%

^{1}H-NMR ($CDCl_3$): δ 2.23 (s, 3H), 2.35 – 2.56 (m, 8H), 2.54 (t, *J* = 5.9 Hz, 2H), 3.45 (dd, *J* = 5.4, 10.7 Hz, 2H), 6.85 (s, 1H), 7.00 (d, *J* = 11.1 Hz, 1H), 7.05 – 7.13 (m, 2H), 7.41 – 7.49 (m, 2H), 8.04 (t, *J* = 8.5 Hz, 1H), 8.67 (d, *J* = 8.4 Hz, 1H), 11.89 (d, *J* = 5.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.23 (CH_2, C-aliphatic), 45.95 (CH_3, C-aliphatic), 52.69 (CH_2, C-aliphatic), 55.12 (CH_2, C-aliphatic), 55.90 (CH_2, C-aliphatic), 109.33(CH, C- aromatic), 116.56 (CH, C-aromatic), 121.41 (C, C-aromatic), 122.43 (CH, C- aromatic), 123.59 (CH, C- aromatic), 126.61 (CH, C- aromatic), 132.44 (CH, C- aromatic), 133.01 (CH, C- aromatic), 139.07 (C, C- aromatic), 151.86 (C,C-aromatic), 159.33 (C, C-aromatic), 160.83 (C, C- aromatic), 161.81 (C, C-aromatic), 168.28 (C, C- aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 57.86, -108.13 ppm.

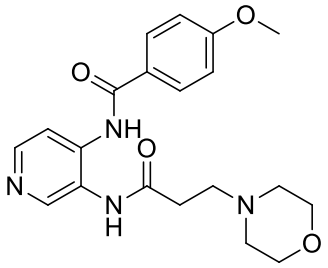
MS (ESI)$^+$: 469.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 69 - 71°C

Synthesis of 4-methoxy-N-(3-(3-morpholinopropanamido)pyridin-4-yl)benzamide (106)

Chemical Formula: $C_{20}H_{24}N_4O_4$

Molecular Weight: 384.43



Procedure: 2

State: white powder

Yield: 55%

^{1}H-NMR ($CDCl_3$): δ 2.46 (d, *J* = 4.2 Hz, 4H), 2.58 (t, *J* = 5.9 Hz, 2H), 3.51 (dd, *J* = 5.3, 11.1 Hz, 2H), 3.62 – 3.70 (m, 4H), 3.81 (s, 3H), 6.88 – 6.99 (m, 2H), 7.04 (s, 1H), 7.19 (s, 1H), 7.94 (dd, *J* = 2.4, 9.4 Hz, 2H), 8.54 (d, *J* = 5.8 Hz, 1H), 8.63 – 8.73 (m, 1H), 12.36 (s, 1H) ppm.

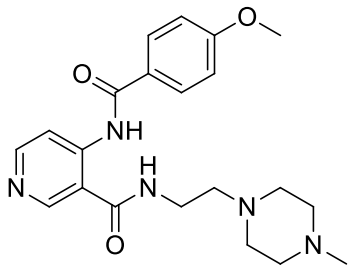
^{13}C-NMR ($CDCl_3$): δ 35.93 (CH_2, C-aliphatic), 53.28 (CH_2, C-aliphatic), 55.49 (CH_3, C-aliphatic), 56.30 (CH_2, C-aliphatic), 66.95 (CH_2, C-aliphatic), 114.16 (CH, C-aromatic), 114.46 (CH, C-aromatic), 126.25 (C, C-aromatic), 129.59 (CH, C-aromatic), 147.22 (C, C-aromatic), 147.94 (CH, C-aromatic), 153.46 (CH, C-aromatic), 163.06 (C, C-aromatic), 165.50 (C, C-aromatic), 165.62 (C, C-aromatic), 167.69 (C, C-aromatic) ppm.

MS (ESI)$^+$: 385.2 $[M+H]^+$

m.p.: (from ethanol/water) 105 – 107 °C

Synthesis of 4-(4-methoxybenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)nicotinamide (107)
Chemical Formula: $C_{21}H_{27}N_5O_3$

Molecular Weight: 397.47



Procedure: 2

State: white powder

Yield: 45%

^{1}H-NMR ($CDCl_3$): δ 2.25 (s, 3H), 2.35 – 2.56 (m, 7H), 2.56 – 2.61 (m, 3H), 3.49 (dd, *J* = 5.6, 10.8 Hz, 2H), 3.81 (s, 3H), 6.91 – 6.96 (m, 2H), 7.12 (s, 1H), 7.89 – 8.00 (m, 2H), 8.53 (d, *J* = 5.8 Hz, 1H), 8.68 (s, 1H), 8.70 (d, *J* = 5.8 Hz, 1H), 12.41 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.16 (CH_2, C-aliphatic), 45.91 (CH_3, C-aliphatic), 52.63 (CH_2, C-aliphatic), 55.09 (CH_2, C-aliphatic), 55.48 (CH_3, C-aliphatic), 55.56 (CH_2, C-aliphatic), 114.16 (CH, C-aromatic), 114.43 (CH, C- aromatic), 114.95 (C, C-aromatic), 126.23 (C, C-aromatic), 129.60 (CH, C- aromatic), 147.25 (C, C- aromatic), 147.99 (CH, C- aromatic), 153.37 (CH, C- aromatic), 163.04 (C, C- aromatic), 165.22 (C, C- aromatic), 167.68 (C, C- aromatic) ppm.

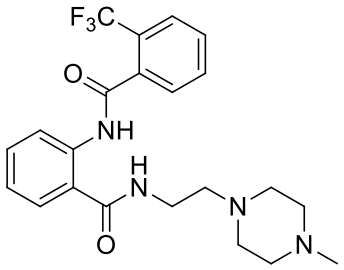
MS (ESI)$^+$: 398.2 $[M+H]^+$

m.p.: (from dichloromethane/*n*-hexane) 83 - 85°C

Synthesis of N-(2-(4-methylpiperazin-1-yl)ethyl)-2-(2-(trifluoromethyl)benzamido)benzamide (108)

Chemical Formula: $C_{22}H_{25}F_3N_4O_2$

Molecular Weight: 434.45



Procedure: 2

State: white powder

Yield: 38 %

^{1}H-NMR ($CDCl_3$): δ 2.24 (s, 3H), 2.35 – 2.47 (m, 7H), 2.51 (dd, *J* = 8.3, 14.2 Hz, 3H), 3.39 (dd, *J* = 5.6, 11.0 Hz, 2H), 6.91 (s, 1H), 7.11 (td, *J* = 1.1, 7.8 Hz, 1H), 7.42 (dd, *J* = 1.3, 7.9 Hz, 1H), 7.46 – 7.52 (m, 2H), 7.56 (t, *J* = 7.3 Hz, 1H), 7.61 (d, *J* = 7.4 Hz, 1H), 7.67 (d, *J* = 7.8 Hz, 1H), 8.71 (d, *J* = 8.3 Hz, 1H), 11.55 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.17 (CH_2, C-aliphatic), 46.00 (CH_3, C-aliphatic), 52.73 (CH_2, C-aliphatic), 55.18 (CH_2, C-aliphatic), 55.80 (CH_2, C-aliphatic), 113.80 (C, C-aromatic), 120.68 (C, C-aromatic), 121.76 (CH, C-aromatic), 123.43 (CH, C-aromatic), 126.46 (CH, C-aromatic), 126.74 (CH, C-aromatic), 126.77 (C, C-aromatic), 128.14 (CH, C-aromatic), 129.99 (CH, C-aromatic), 132.17 (CH, C-aromatic), 132.72 (CH, C-aromatic), 139.50 (C, C-aromatic), 166.14 (C, C-aromatic), 168.65 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -58.93 ppm.

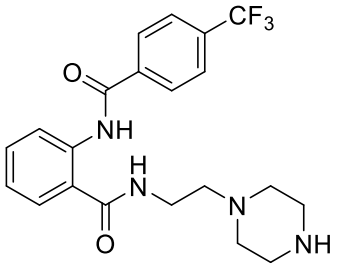
MS (ESI)$^+$: 435.2 $[M+H]^+$

m.p.: (from ethanol/water) 124 - 126°C

Synthesis of *N*-(2-(piperazin-1-yl)ethyl)-2-(4-(trifluoromethyl)benzamido)benzamidee (109)

Chemical Formula: $C_{21}H_{23}F_3N_4O_2$

Molecular Weight: 420.44



Procedure: 2

State: white powder

Yield: 18 %

^{1}H-NMR ($CDCl_3$): δ 1.67 (s, 1H), 2.42 – 2.57 (m, 4H), 2.59 – 2.72 (m, 2H), 2.95 (t, J = 4.8 Hz, 4H), 3.55 (dt, J = 12.7, 25.5 Hz, 2H), 7.00 – 7.19 (m, 1H), 7.13 (s, 1H), 7.49 – 7.64 (m, 2H), 7.80 (d, J = 8.2 Hz, 2H), 8.10-8.18 (m, 2H), 8.86 (dt, J = 3.3, 6.5 Hz, 1H), 12.50 (d, J = 62.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.07 (CH_2, C-aliphatic), 46.07 (CH_2, C-aliphatic), 53.92 (CH_2, C-aliphatic), 56.41 (CH_2, C-aliphatic), 120.46 (C, C-aromatic), 121.58 (CH, C-aromatic), 123.26 (CH, C-aromatic), 125.82 (CH, C-aromatic), 126.54 (CH, C-aromatic), 127.87 (CH, C-aromatic), 132.89 (CH, C-aromatic), 138.60 (C, C-aromatic), 140.01 (C, C-aromatic), 164.70 (C, C-aromatic), 166.14 (C, C-aromatic), 168.65 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -62.98 ppm.

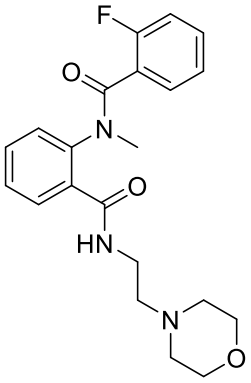
MS(ESI)$^+$: 421.2 $[M+H]^+$

m.p.: (from ethanol/water) 71 – 73 °C

Synthesis of 2-fluoro-N-methyl-N-(2-((2-morpholinoethyl)carbamoyl)phenyl)benzamide (110)

Chemical Formula: $C_{21}H_{24}FN_3O_3$

Molecular Weight: 385.44



Procedure: A solution was prepared by adding compound **82** (1 equivalent), HATU (1.2 equivalent), DIPEA (2.1 equivalent) and 2-morpholinoethan-1-amine (1.2 equivalent) in DMF. The reaction was stirred at 25 °C for 8 hours. The reaction mixture was neutralised with NaOH 1M, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated to give a yellow oil which was purified by silica gel column chromatography using ethyl acetate/*n*-hexane as eluent.

State: white powder

Yield: 28 %

^{1}H-NMR ($CDCl_3$): δ 2.40 – 2.50 (m, 4H), 2.53 (t, J = 5.6 Hz, 2H), 3.38 (d, J = 2.8 Hz, 2H), 3.49 – 3.55 (m, 4H), 3.58 – 3.63 (s, 3H), 6.73 (dt, J = 5.9, 9.3 Hz, 1H), 6.87 – 6.98 (m, 1H), 7.00 – 7.1 (m, 2H), 7.11 – 7.19 (m, 1H), 7.30 – 7.42 (m, 2H), 7.58 – 7.67 (m, 1H), 8.02 (td, J = 1.9, 7.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.07 (CH_2, C-aliphatic), 38.20 (CH_3, C-aliphatic), 53.30 (CH_2, C-aliphatic), 56.76 (CH_2, C-aliphatic), 66.73 (CH_2, C-aliphatic), 109.23 (C, C-aromatic), 115.96 (CH, C-aromatic), 116.30 (CH, C-aromatic), 120.97 (CH, C-aromatic), 124.72 (CH, C-aromatic), 131.80 (CH, C-aromatic), 133.34 (CH, C-aromatic), 123.30 (CH, C-aromatic),126.54 (CH, C-aromatic), 159.63 (C, C-aromatic), 160.08 (C, C-aromatic), 161.60 (C, C-aromatic), 1663.58 (C, C-aromatic), 164.81 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 113.08 ppm.

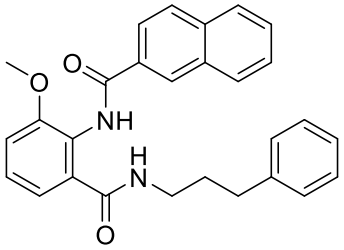
MS(ESI)$^+$: 386.2 $[M+H]^+$

m.p.: 101 – 103 °C

Synthesis of *N*-(2-methoxy-6-(3-phenylpropylcarbamoyl)phenyl)-2-naphthamide (111)

Chemical Formula: $C_{28}H_{26}N_2O_3$

Molecular Weight: 438.51



Procedure: 2

State: white powder

Yield: 45 %

^{1}H-NMR ($CDCl_3$): δ 1.81 – 1.93 (m, 2H), 2.60 – 2.66 (m, 2H), 3.40 (dd, J = 6.8, 13.1 Hz, 2H), 3.90 (s, 3H), 6.55 (s, 1H), 7.07 (t, J = 7.0 Hz, 4H), 7.16 (dq, J = 7.0, 14.2 Hz, 2H), 7.26 – 7.32 (m, 2H), 7.60 (ddd, J = 6.8, 13.7, 14.9 Hz, 2H), 7.89 – 7.99 (m, 3H), 8.00 (dd, J = 11.9, 13.5 Hz, 1H), 8.52 (s, 1H), 8.91 (s, 1H) ppm.

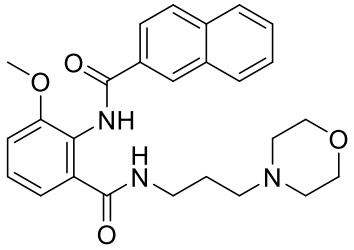
^{13}C-NMR ($CDCl_3$): δ 30.97 (CH_2, C-aliphatic), 32.26 (CH_2, C-aliphatic), 39.56 (CH_2, C-aliphatic), 56.18 (CH_3, C-aliphatic), 113.49 (CH, C-aromatic), 119.44 (CH, C-aromatic), 124.01 (CH, C-aromatic), 125.89 (CH, C-aromatic), 126.80 (CH, C-aromatic), 127.40 (CH, C-aromatic), 127.78 (CH, C-aromatic), 127.92 (CH, C-aromatic), 128.31 (CH, C-aromatic), 128.37 (CH, C-aromatic), 128.48 (CH, C-aromatic) 128.62 (CH, C-aromatic), 129.22 (CH, C-aromatic), 131.20 (C, C-aromatic), 132.67 (C, C-aromatic), 133.64 (C, C-aromatic), 135.07 (C, C-aromatic), 141.34 (C, C-aromatic), 154.32 (C, C-aromatic), 167.08 (C, C-aromatic), 168.34 (C, C-aromatic) ppm.

MS (ESI)$^+$: 439.2 $[M+H]^+$

m.p.: (from methanol/water) 152-154 °C

Synthesis of *N*-(2-methoxy-6-(3-morpholinopropylcarbamoyl)phenyl)-2-naphthamide (112)
Chemical Formula: $C_{26}H_{29}N_3O_4$

Molecular Weight: 447.53



Procedure: 2

State: white powder

Yield: 38 %

^{1}H-NMR ($CDCl_3$): δ 1.61-1.64 (m, 3H), 2.32 (s, 3H), 2.39 (t, J = 6.0 Hz, 2H), 3.38 (dd, J = 5.6, 11.3 Hz, 2H), 3.50-3.53 (m, 4H), 3.84 (s, 3H), 7.05 (dd, J = 8.0, 15.8 Hz, 2H), 7.21 (s, 1H), 7.46-7.53 (m, 2H), 7.81-7.93 (5H, m), 8.45 (1H, s), 9.46 (1H, s) ppm.

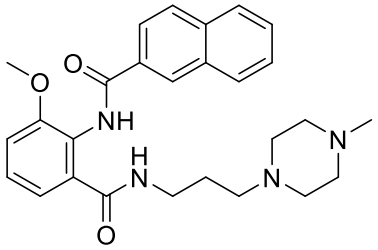
^{13}C-NMR ($CDCl_3$): δ 24.30 (CH_2, C-aliphatic), 40.19 (CH_2, C-aliphatic), 53.67 (CH_2, C-aliphatic), 56.27 (CH_3, C-aliphatic), 58.18 (CH_2, C-aliphatic), 66.89 (CH_2, C-aliphatic), 114.09 (CH, C-aromatic), 119.02 (CH, C-aromatic), 124.20 (CH, C-aromatic), 125.59 (C, C-aromatic), 126.56 (CH, C-aromatic), 126.72 (CH, C-aromatic), 127.75 (CH, C-aromatic), 127.82 (CH, C-aromatic), 128.49 (CH, C-aromatic), 128.51 (CH, C-aromatic), 129.21 (CH, C-aromatic) 131.57 (C, C-aromatic), 131.68 (C, C-aromatic), 132.70 (C, C-aromatic), 135.01 (C, C-aromatic), 154.44 (C, C-aromatic), 166.10 (C, C-aromatic), 168.45 (C, C-aromatic) ppm.

MS (ESI)$^+$: 448.2 $[M+H]^+$

m.p.: (from methanol/water) 140-142 °C

Synthesis of *N*-(2-methoxy-6-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)-2-naphthamide (113)

Chemical Formula: $C_{24}H_{31}N_3O_6$
Molecular Weight: 457.53



Procedure: 2

State: white powder

Yield: 71%

^{1}H-NMR ($CDCl_3$): δ 1.73 (dt, J = 6.0, 12.0 Hz, 3H), 2.27 (s, 3H), 2.35 (bs, 6H), 2.51 – 2.56 (m, 3H), 3.47 (dd, J = 5.8, 11.2 Hz, 2H), 3.95 (s, 3H), 7.15 (d, J = 8.3 Hz, 1H), 7.20 (dd, J = 1.2, 7.8 Hz, 1H), 7.24 – 7.34 (m, 1H), 7.54 – 7.63 (m, 2H), 7.91 (d, J = 7.9 Hz, 1H), 7.95 (d, J = 8.6 Hz, 1H), 8.00 (d, J = 7.9 Hz, 1H), 8.06 (dd, J = 1.8, 8.6 Hz, 1H), 8.26 (s, 1H), 8.55 (s, 1H), 9.77 (s, 1H) ppm.

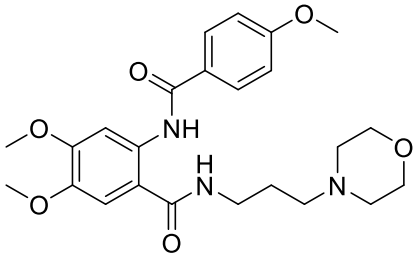
^{13}C-NMR ($CDCl_3$): δ 24.31 (CH_2, C-aliphatic), 40.44 (CH_2, C-aliphatic), 45.93 (CH_3, C-aliphatic), 53.07 (CH_2, C-aliphatic), 54.94 (CH_2, C-aliphatic), 56.28 (CH_3, C-aliphatic), 57.79 (CH_2, C-aliphatic), 114.25 (CH, C-aromatic), 119.38 (CH, C-aromatic), 124.25 (CH, C-aromatic), 125.99 (C, C-aromatic), 126.29 (CH, C-aromatic), 126.65 (CH, C-aromatic), 127.74 (CH, C-aromatic), 128.47 (CH, C-aromatic), 128.47 (CH, C-aromatic), 128.50 (CH, C-aromatic), 129.23 (CH, C-aromatic), 131.21 (C, C-aromatic), 131.68 (C, C-aromatic), 132.73 (C, C-aromatic), 135.01 (C, C-aromatic), 154.46 (C, C-aromatic), 165.94 (C, C-aromatic), 168.42 (C, C-aromatic) ppm.

MS (ESI)$^+$: 448.2 $[M+H]^+$

m.p.: (from methanol/water) 111-113 °C

Synthesis of 4,5-dimethoxy-2-(4-methoxybenzamido)-N-(3-morpholinopropyl)benzamide (114)
Chemical Formula: $C_{27}H_{32}N_4O_3$

Molecular Weight: 447.53



Procedure: 2

State: white powder

Yield: 39 %

^{1}H-NMR ($CDCl_3$): δ 1.73 (dt, J = 6.0, 12.0 Hz, 3H), 2.27 (s, 3H), 2.35 (bs, 6H), 2.51 – 2.56 (m, 3H), 3.47 (dd, J = 5.8, 11.2 Hz, 2H), 3.95 (s, 3H), 7.15 (d, J = 8.3 Hz, 1H), 7.20 (dd, J = 1.2, 7.8 Hz, 1H), 7.24 – 7.34 (m, 1H), 7.54 – 7.63 (m, 2H), 7.91 (d, J = 7.9 Hz, 1H), 7.95 (d, J = 8.6 Hz, 1H), 8.00 (d, J = 7.9 Hz, 1H), 8.06 (dd, J = 1.8, 8.6 Hz, 1H), 8.26 (s, 1H), 8.55 (s, 1H), 9.77 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.22 (CH_2, C-aliphatic), 40.38 (CH_2, C-aliphatic), 53.84 (CH_2, C-aliphatic), 55.44 (CH_3, C-aliphatic), 56.07 (CH_3, C-aliphatic), 57.60 (CH_3, C-aliphatic), 58.42 (CH_2, C-aliphatic), 66.77 (CH_2, C-aromatic), 104.91 (CH, C-aromatic), 111.78 (CH, C-aromatic), 113.99 (CH, C-aromatic), 127.30 (C, C-aromatic), 129.23 (CH, C-aromatic), 136.58 (C, C-aromatic), 143.99 (C, C-aromatic), 153.04 (C, C-aromatic), 162.45 (C, C-aromatic), 165.19 (C, C-aromatic), 165.55 (C, C-aromatic), 169.09 (C, C-aromatic) ppm.

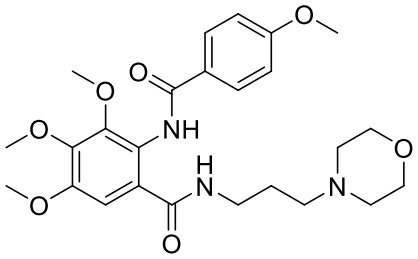
MS (ESI)$^+$: 448.2 $[M+H]^+$

m.p.: (from acetone/*n*-hexane) 102-104 °C

Synthesis of 3, 4, 5-trimethoxy-2-(4-methoxybenzamido)-N-(3-morpholinopropyl)benzamide (115)

Chemical Formula: $C_{25}H_{33}N_3O_7$

Molecular Weight: 487.52



Procedure: 2

State: white powder

Yield: 63 %

^{1}H-NMR ($CDCl_3$): δ 1.58 (dt, J = 6.6, 13.2 Hz, 2H), 2.29 (dd, J = 9.5, 16.2 Hz, 4H), 3.33 (dd, J = 6.4, 12.1 Hz, 2H), 3.41 – 3.64 (m, 4H), 3.81 (s, 3H), 3.82 (s, 3H), 3.83 (s, 3H), 3.85 (s, 3H), 6.75 – 6.88 (m, 2H), 6.89 – 6.98 (m, 4H), 7.67 – 7.89 (m, 4H), 8.47 (s, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 25.36 (CH_2, C-aliphatic), 39.23 (CH_2, C-aliphatic), 53.59 (CH_2, C-aliphatic), 55.50 (CH_3, C-aliphatic), 56.58 (CH_3, C-aliphatic), 57.18 (CH_3, C-aliphatic), 61.01 (CH_3, C-aliphatic), 61.07 (CH_2, C-aliphatic), 66.94 (CH_2, C-aliphatic), 106.66 (CH, C-aromatic), 113.96 (CH, C-aromatic), 122.67 (C, C-aromatic), 126.22 (C, C-aromatic), 128.41 (C, C-aromatic), 129.49 (CH, C-aromatic), 144.66 (C, C-aromatic), 149.46 (C, C-aromatic), 152.01 (C, C-aromatic), 162.75 (C, C-aromatic), 166.96 (C, C-aromatic), 167.99 (C, C-aromatic) ppm.

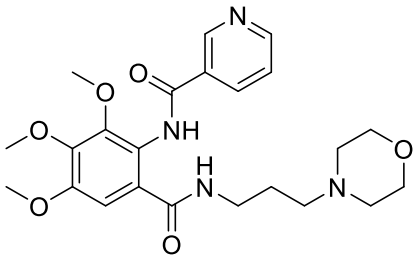
MS (ESI)$^+$: 488.2 $[M+H]^+$

m.p.: (from acetone/*n*-hexane) 121-123 °C

Synthesis of *N*-(2,3,4-trimethoxy-6-((3-morpholinopropyl)carbamoyl)phenyl)nicotinamide (116)

Chemical Formula: $C_{23}H_{30}N_4O_6$

Molecular Weight: 458.52



Procedure: 2

State: white powder

Yield: 23 %

^{1}H-NMR ($CDCl_3$): δ 1.70 – 1.76 (m, 2H), 2.43 (s, 2H), 2.48 (t, J = 5.5 Hz, 2H), 3.42 – 3.52 (m, 8H), 3.66 (t, J = 4.4 Hz, 3H), 3.94 (s, 3H), 3.96 (s, 3H), 6.85 (s, 1H), 7.45 (dd, J = 4.8, 7.9 Hz, 1H), 7.52 (d, J = 26.4 Hz, 1H), 8.22 – 8.29 (m, 1H), 8.80 (dd, J = 4.8, 1.5 Hz, 1H), 9.25 – 9.13 (m, 2H) ppm.

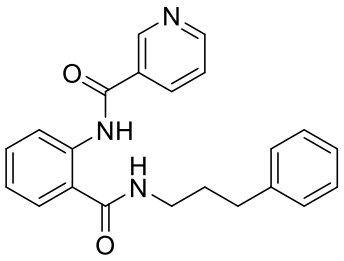
^{13}C-NMR ($CDCl_3$): δ 24.82 (CH_2, C-aliphatic), 39.85 (CH_2, C-aliphatic), 53.69 (CH_2, C-aliphatic), 56. (CH_3, C-aliphatic), 57.73 (CH_2, C-aliphatic), 60.99 (CH_3, C-aliphatic), 61.02 (CH_3, C-aliphatic), 66.93 (CH_3, C-aliphatic), 106.38 (CH, C-aromatic), 123.03 (C, C-aromatic), 123.50 (CH, C-aromatic), 126.44 (C, C-aromatic), 130.09 (C, C-aromatic), 135.28 (CH, C-aromatic), 144.86 (C, C-aromatic), 148.95 (CH, C-aromatic), 149.11 (C, C-aromatic), 151.64 (C, C-aromatic), 152.63 (CH, C-aromatic), 164.99 (C, C-aromatic), 168.06 (C, C-aromatic) ppm.

MS(ESI)$^+$: 459.2 $[M+H]^+$

m.p.: (from acetone/*n*-hexane) 120-122 °C

Synthesis of *N*-(2-((3-phenylpropyl)carbamoyl)phenyl)nicotinamide (117)
Chemical Formula: $C_{23}H_{30}N_4O_6$

Molecular Weight: 458.52



Procedure: 2

State: white powder

Yield: 65 %

^{1}H-NMR ($CDCl_3$): δ 1.80 – 2.00 (m, 2H), 2.59 – 2.77 (m, 2H), 3.45 (dd, J = 6.9, 12.7 Hz, 2H), 6.18 (s, 1H), 7.00 (tt, J = 4.2, 8.4 Hz, 1H), 7.13 – 7.17 (m, 4H), 7.22 – 7.25 (m, 3H), 7.34 – 7.40 (m, 1H), 7.43 – 7.48 (m, 1H), 8.14 – 8.31 (m, 1H), 8.43 – 8.83 (m, 2H), 9.22 (s, 1H), 12.33 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.77 (CH_2, C-aliphatic), 33.64 (CH_2, C-aliphatic), 39.95 (CH_2, C-aliphatic), 120.19 (CH, C-aromatic), 121.61 (CH, C-aromatic), 123.16 (C, C-aromatic), 123.48 (CH, C-aromatic), 126.34 (CH, C-aromatic), 128.40 (CH, C-aromatic), 128.73 (CH, C-aromatic), 130.11 (C,C-aromatic), 130.47 (CH, C-aromatic), 132.85 (CH, C-aromatic), 134.86 (CH, C-aromatic), 139.73 (C, C-aromatic), 141.21 (C, C-aromatic), 149.20 (CH, C-aromatic), 152.48 (CH, C-aromatic), 163.75 (C, C-aromatic), 169.01 (C, C-aromatic) ppm.

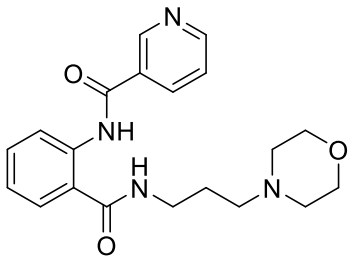
MS(ESI)$^+$: 457.3 $[M-H]^+$

m.p.: (from ethanol/water) 74-76 °C

Synthesis of *N*-(2-((3-morpholinopropyl)carbamoyl)phenyl)nicotinamide (118)

Chemical Formula: $C_{20}H_{24}N_4O_3$

Molecular Weight: 368.18



Procedure: 2

State: white powder

Yield: 79 %

^{1}H-NMR ($CDCl_3$): δ 1.75 (dt, J = 5.8, 11.6 Hz, 2H), 2.46 (s, 4H), 2.52 – 2.56 (m, 2H), 3.52 (dd, J = 5.9, 10.6 Hz, 2H), 3.64 (t, J = 4.5 Hz, 4H), 7.10 (td, J = 1.1, 7.8 Hz, 1H), 7.37 (ddd, J = 0.7, 4.8, 7.9 Hz, 1H), 7.47 – 7.51 (m, 1H), 7.54 – 7.58 (m, 1H), 8.25 (ddd, J = 1.7, 2.2, 8.0 Hz, 1H), 8.67 (s, 1H), 8.70 (dd, J = 1.6, 4.8 Hz, 1H), 8.76 (dd, J = 1.0, 8.4 Hz, 1H), 9.24 (d, J = 1.8 Hz, 1H), 12.62 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.41 (CH_2, C-aliphatic), 33.58 (CH_2, C-aliphatic), 39.82 (CH_2, C-aliphatic), 57.62 (CH_2, C-aliphatic), 60.10 (CH_2, C-aliphatic), 124.46 (CH, C-aromatic), 128.52 (CH, C-aromatic), 128.73 (CH, C-aromatic), 130.07 (C,C-aromatic), 130.47 (CH, C-aromatic), 132.85 (CH, C-aromatic), 134.86 (CH, C-aromatic), 139.73 (C, C-aromatic), 141.21 (C, C-aromatic), 149.20 (CH, C-aromatic), 152.48 (CH, C-aromatic), 163.75 (C, C-aromatic), 169.01 (C, C-aromatic) ppm.

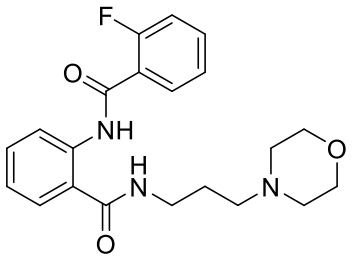
MS(ESI)$^+$: 367.2 $[M-H]^+$

m.p.: (from acetone/*n*-hexane) 120-122 °C

Synthesis of 2-fluoro-*N*-(2-((3-morpholinopropyl)carbamoyl)phenyl)benzamide (119)

Chemical Formula: $C_{21}H_{24}FN_3O_3$

Molecular Weight: 385.18



Procedure: 2

State: white powder

Yield: 75 %

^{1}H-NMR ($CDCl_3$): δ 1.73 (dt, J = 5.8, 11.4 Hz, 2H), 2.45 (bs, 4H), 2.50 – 2.59 (m, 2H), 3.50 (dd, J = 5.7, 10.8 Hz, 2H), 3.62 (s, 4H), 7.04 – 7.15 (m, 2H), 7.19 – 7.21 (m, 1H), 7.38 – 7.66 (m, 4H), 7.97 (td, J = 1.8, 7.7 Hz, 1H), 8.47 (s, 1H), 8.69 (d, J = 7.9 Hz, 1H), 11.95 (d, J = 6.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.41 (CH_2, C-aliphatic), 40.95 (CH_2, C-aliphatic), 53.79 (CH_2, C-aliphatic), 58.94 (CH_2, C-aliphatic), 66.83 (CH_2, C-aliphatic), 116.58 (CH, C-aromatic, d, J = 89.49 Hz), 121.86 (C, C-aromatic), 122.40 (CH, C-aromatic), 123.14 (CH, C-aromatic), 124.60 (CH, C-aromatic), 126.84 (CH, C-aromatic), 131.49 (CH, C-aromatic), 132.34 (CH, C-aromatic),133.30 (CH, C-aromatic), 139.20 (C, C aromatic), 159.37 (C, C-aromatic), 161.37 (C, C-aromatic), 162.38 (C, C-aromatic), 168.69 (C, C-aromatic) ppm.

19F-NMR ($CDCl_3$): δ -112.58 ppm.

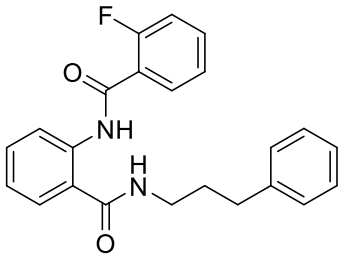
MS(ESI)$^+$: 386.2 $[M+H]^+$

m.p.: 77 – 79 °C

Synthesis of 2-fluoro-*N*-(2-((3-phenylpropyl)carbamoyl)phenyl)benzamide (120)

Chemical Formula: $C_{23}H_{21}FN_2O_2$

Molecular Weight: 376.43



Procedure: 2

State: white powder

Yield: 63 %

^{1}H-NMR ($CDCl_3$): δ 2.65 – 2.72 (t, J = 7.3 Hz, 2H), 2.77 (t, J = 7.2 Hz, 2H), 3.53 (dd, J = 6.9, 12.8 Hz, 2H), 7.11 (td, J = 1.1, 7.7 Hz, 1H), 7.22 (dt, J = 7.0, 14.0 Hz, 5H), 7.26 – 7.30 (m, 4H), 7.48 – 7.57 (m, 2H), 8.07 (td, J = 5.3, 10.5 Hz, 1H), 8.74 (d, J = 7.8 Hz, 1H), 11.70 (s, 1H) ppm

^{13}C-NMR ($CDCl_3$): δ 34.73 (CH_2, C-aliphatic), 39.42 (CH_2, C-aliphatic), 41.54 (CH_2, C-aliphatic), 116.44 (CH, C-aromatic), 116.63 (CH, C-aromatic), 122.49 (CH, C-aromatic), 123.30 (CH, C-aromatic), 124.62 (CH, C-aromatic), 125.83 (CH, C-aromatic), 126.17 (CH, C-aromatic), 126.39 (CH, C-aromatic), 128.37 (CH, C-aromatic), 128.65 (CH, C-aromatic), 131.54 (CH, C-aromatic), 132.27 (CH, C-aromatic), 133.34 (C, C-aromatic, d, J = 8.9 Hz), 141.28 (C, C-aromatic), 141.97 (C, C-aromatic), 159.37 (C, C-aromatic), 161.37 (C, C-aromatic), 162.32 (C, C-aromatic), 168.65 (C, C-aromatic) ppm.

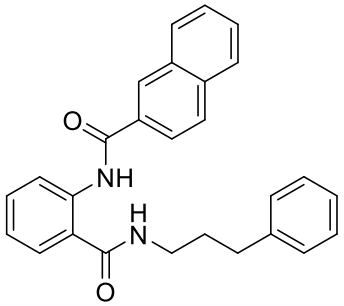
^{19}F-NMR ($CDCl_3$): δ -112.40 ppm.

MS (ESI)$^+$: 399.1 $[M+Na]^+$

m.p.: (from dichloromethane/ *n*-hexane) 61 – 63 °C

Synthesis of *N*-(2-((3-phenylpropyl)carbamoyl)phenyl)-2-naphthamide (121)

Chemical Formula: $C_{27}H_{24}N_2O_2$
Molecular Weight: 408.50



Procedure: 2

State: white powder

Yield: 57 %

^{1}H-NMR ($CDCl_3$): δ 1.98 – 2.10 (t, J = 7.26 Hz, 2H), 2.62 – 2.73 (t, J = 7.75 Hz, 2H), 3.56 (dd, J = 6.9, 12.8 Hz, 2H), 6.32 (s, 1H), 7.09 (td, J = 1.1, 7.8 Hz, 1H), 7.52 – 7.66 (m, 4H), 7.93 (d, J = 7.8 Hz, 1H), 7.99 (d, J = 8.6 Hz, 1H), 8.05 (d, J = 7.8 Hz, 1H), 8.11 (dd, J = 1.8, 8.6 Hz, 4H), 8.60 (s, 1H), 8.83 – 8.87 (m, 3H), 12.29 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.86 (CH_2, C-aliphatic), 33.61 (CH_2, C-aliphatic), 39.90 (CH_2, C-aliphatic), 120.67 (C, C-aromatic), 122.75 (CH, C-aromatic), 123.69 (CH, C-aromatic), 125.83 (CH, C-aromatic), 126.30 (CH, C-aromatic), 126.63 (CH, C-aromatic), 127.75 (CH, C-aromatic), 126.21 (CH, C-aromatic), 127.79 (CH, C-aromatic), 128.39 (CH, C-aromatic), 128.41 (CH, C-aromatic), 128.63 (CH, C-aromatic), 128.65 (CH, C-aromatic), 129.41 (CH, C-aromatic), 132.14 (C, C-aromatic), 132.63 (CH, C-aromatic), 132.81 (C, C-aromatic), 134.98 (C, C-aromatic), 139.96 (C, C-aromatic), 142.25 (C, C-aromatic), 165.68 (C, C-aromatic), 169.16 (C, C-aromatic) ppm.

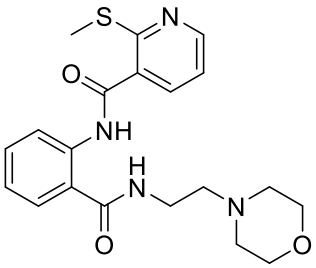
MS (ESI)$^+$: 431.2 $[M+Na]^+$

m.p.: (from dichloromethane/ *n*-hexane) 99 – 101 °C

Synthesis of 2-(methylthio)-*N*-(2-((2-morpholinoethyl)carbamoyl)phenyl)nicotinamide (122)

Chemical Formula: $C_{20}H_{24}N_4O_3S$

Molecular Weight: 400.50



Procedure: 2

State: white powder

Yield: 76 %

^{1}H-NMR ($CDCl_3$): δ 2.44 (d, J = 4.1 Hz, 4H), 2.53 (dd, J = 9, 14.9 Hz, 2H), 3.06 (s, 3H), 3.44 (dd, J = 5.4, 11.2 Hz, 2H), 3.63 – 3.71 (m, 4H), 6.88 (s, 1H), 7.04 (dd, J = 4.8, 7.7 Hz, 1H), 7.09 (td, J = 1.1, 7.8 Hz, 1H), 7.40 – 7.52 (m, 2H), 7.88 (dd, J = 1.7, 7.7 Hz, 1H), 8.49 (dd, J = 1.7, 4.8 Hz, 1H), 8.72 (dd, J = 0.9, 8.4 Hz, 1H), 11.91 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 13.90 (CH_3, C-aliphatic), 35.94 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 56.43 (CH_2, C-aliphatic), 66.99 (CH_2, C-aliphatic), 118.47 (CH, C-aromatic), 120.43 (C, C-aromatic), 121.87 (CH, C-aromatic), 123.31 (CH, C-aromatic), 126.43 (CH, C-aromatic), 129.00 (C, C-aromatic), 132.82 (CH, C-aromatic), 134.83 (CH, C-aromatic), 139.71 (C, C-aromatic), 150.74 (CH, C-aromatic), 160.53 (C, C-aromatic), 165.16 (C, C-aromatic), 168.96 (C, C-aromatic) ppm.

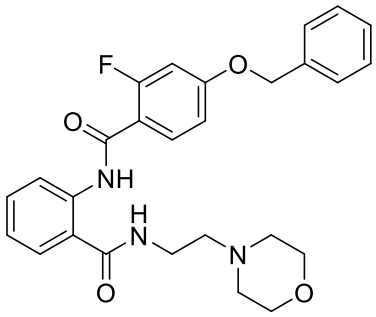
MS(ESI)$^-$: 399.3 $[M-H]^-$

m.p.: (from dichloromethane/ *n*-hexane) 110 – 112 °C

Synthesis of 4-(benzyloxy)-2-fluoro-*N*-(2-((2-morpholinoethyl)carbamoyl)phenyl)benzamide (123)

Chemical Formula: $C_{27}H_{28}FN_3O_4$

Molecular Weight: 477.54



Procedure: 2

State: white powder

Yield: 58 %

^{1}H-NMR ($CDCl_3$): δ 2.53 (s, 4H), 2.62 (s, 2H), 3.52 (d, J = 5.4 Hz, 2H), 3.70 (s, 4H), 5.02 (d, J = 21.9 Hz, 2H), 6.68 (dd, J = 2.4, 13.3 Hz, 1H), 6.80 (dd, J = 2.4, 8.8 Hz, 1H), 7.05 (t, J = 7.2 Hz, 6H), 7.37 – 7.26 (m, 2H), 7.45 (dd, J = 8.4, 11.4 Hz, 1H), 7.96 (t, J = 8.9 Hz, 1H), 8.64 (d, J = 8.3 Hz, 1H), 11.61 (d, J = 8.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.63 (CH_2, C-aliphatic), 53.10 (CH_2, C-aliphatic), 56.81 (CH_2, C-aliphatic), 66.22 (CH_2, C-aliphatic), 70.54 (CH_2, C-aliphatic), 102.70 (CH, C-aromatic), 102.92 (CH, C-aromatic), 111.42 (CH, C-aromatic), 115.04 (C, C-aromatic), 121.74 (C, C-aromatic), 122.43 (CH, C-aromatic), 123.19 (CH, C-aromatic), 126.80 (CH, C-aromatic), 127.07 (CH, C-aromatic), 128.39 (CH, C-aromatic), 128.76 (CH, C-aromatic), 132.99 (CH, C-aromatic), 135.79 (C, C-aromatic), 139.09 (C, C-aromatic), 160.46 (C, C-aromatic), 161.95 (C, C-aromatic), 162.46 (C, C-aromatic), 162.73 (C, C-aromatic), 162.82 (C, C-aromatic), 168.97 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.22 ppm.

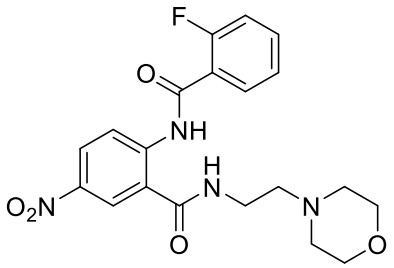
MS(ESI)$^-$: 476.22 $[M-H]^-$

m.p.: (from dichloromethane/ *n*-hexane) 144-146 °C

Synthesis of 2-(2-fluorobenzamido)-*N*-(2-morpholinoethyl)-5-nitrobenzamide (124)

Chemical Formula: $C_{20}H_{21}FN_4O_5$

Molecular Weight: 416.41



Procedure: 2

State: yellow powder

Yield: 34%

^{1}H-NMR ($CDCl_3$): δ 2.43 (d, J = 12.9 Hz, 4H), 2.49 – 2.55 (m, 2H), 3.39 – 3.47 (m, 2H), 3.50 – 3.56 (m, 4H), 6.95 – 7.13 (m, 1H), 7.39 – 7.51 (m, 1H), 8.47 (dd, J = 2.7, 9.3 Hz, 1H), 8.62 (dd, J = 2.7, 14.8 Hz, 1H), 8.69 (d, J = 2.7 Hz, 1H), 8.84 (dd, J = 9.4, 11.9 Hz, 1H), 9.07 (t, J = 5.5 Hz, 1H), 12.44 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.86 (CH_2, C-aliphatic), 50.77 (CH_2, C-aliphatic), 53.20 (CH_2, C-aliphatic), 56.65 (CH_2, C-aliphatic), 113.95 (CH, C-aromatic), 116.47 (C, C-aromatic), 122.03 (CH, C-aromatic), 123.71 (CH, C-aromatic), 124.90 (CH, C-aromatic), 127.78 (CH, C-aromatic), 132.18 (CH, C-aromatic), 148.45 (C, C-aromatic), 153.65 (C, C-aromatic), 159.39 (CH, C-aromatic), 161.39 (C, C-aromatic), 162.69 (C, C-aromatic), 167.16 (C, C-aromatic), 168.28 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 111.94 ppm.

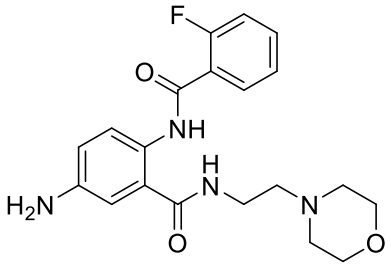
MS(ESI)$^-$: 415.2 $[M-H]^-$

m.p.: (from dichloromethane/ *n*-hexane) 170 – 172 °C

Synthesis of 5-amino-2-(2-fluorobenzamido)-N-(2-morpholinoethyl)benzamide (125)

Chemical Formula: $C_{20}H_{23}FN_4O_3$

Molecular Weight: 386.43



Procedure: A solution of compound **124** (1 equivalent), tin (II) chloride (4 equivalent) in ethyl acetate (1 mL/mmol) was heated at 110 °C for 16 hours. The reaction mixture was poured into sodium bicarbonate solution and the powder was filtrated under reduced pressure. The filtrate was diluted with water, extracted with ethyl acetate and washed with brine. The crude compound was used for the next step of reaction without further purification.

State: yellow powder

Yield: 59%

^{1}H-NMR ($CDCl_3$): δ 2.45 (s, 4H), 2.53 (dd, J = 14.2, 20.2 Hz, 2H), 3.47 (t, J = 5.1 Hz, 2H), 3.61 – 3.70 (m, 4H), 4.02 (bs, 2H), 6.61– 6.78 (m, 2H), 6.84 (s, 1H), 7.10 (ddd, J = 0.8, 8.3, 11.4 Hz, 2H), 7.38 – 7.47 (m, 1H), 7.98 (td, J = 1.8, 7.8 Hz, 1H), 8.34 (d, J = 8.8 Hz, 1H), 11.16 (d, J = 8.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.98 (CH_2, C-aliphatic), 53.56 (CH_2, C-aliphatic), 56.79 (CH_2, C-aliphatic), 66.83 (CH_2, C-aliphatic), 108.00 (CH, C-aromatic), 116.52 (C, C-aromatic), 124.59 (CH, C-aromatic), 126.27 (CH, C-aromatic), 128.28 (CH, C-aromatic), 132.13 (CH, C-aromatic), 133.18 (CH, C-aromatic), 141.92 (CH, C-aromatic), 144.48 (C, C-aromatic), 145.25 (C, C-aromatic), 159.35 (C, C-aromatic), 158.60 (C, C-aromatic), 161.34 (C, C-aromatic), 168.72 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 112.66 ppm.

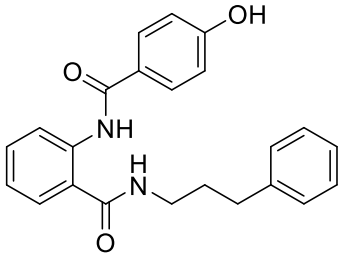
MS(ESI)$^-$: 385.23 $[M-H]^-$

m.p.: (from dichloromethane/ *n*-hexane) 141 – 143 °C

Synthesis of 2-(4-hydroxybenzamido)-N-(3-phenylpropyl)benzamide (126)

Chemical Formula: $C_{23}H_{22}N_2O_3$

Molecular Weight: 374.44



Procedure: A solution was prepared by adding the starting material (1 equivalent), BBr_3 (3 equivalent), anhydrous dichloromethane (2 mL / mmol) at -78 °C. The reaction mixture was stirred for 16 hours and left to reach 25 °C.

The reaction mixture was quenched with iced water, extracted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered and evaporated to give the desired product.

State: colourless oil

Yield: 84%

^{1}H-NMR ($CDCl_3$): δ 2.43 (m, 2H), 2.50 – 2.55 (m, 2H), 3.45 (dd, J = 4.9, 10.8 Hz, 2H), 6.79 – 6.71 (m, 1H), 7.10 (ddd, J = 11.4, 8.3, 0.9 Hz, 2H), 7.21 – 7.17 (m, 5H), 7.46 – 7.35 (m, 2H), 7.96 (tt, J = 60.7, 30.3 Hz, 2H), 8.34 (d, J = 8.8 Hz, 2H), 8.90 – 8.59 (m, 1H), 11.15 (d, J = 8.4 Hz, 1H), ppm.

^{13}C-NMR ($CDCl_3$): δ 31.12 (CH_2, C-aliphatic), 33.52 (CH_2, C-aliphatic), 39.53 (CH_2, C-aliphatic), 115.71 (CH, C-aromatic), 117.24 (C, C-aromatic), 117.76 (CH, C-aromatic), 121.05 (C, C-aromatic), 121.63 (CH, C-aromatic), 122.67 (CH, C-aromatic), 126.08 (CH, C-aromatic), 127.14 (CH, C-aromatic), 128.42 (CH, C-aromatic), 128.58 (CH, C-aromatic), 132.30 (CH, C-aromatic), 141.46 (C, C-aromatic), 160.21 (C, C-aromatic), 161.66 (C, C-aromatic), 169.37 (C, C-aromatic), 171.37 (C, C-aromatic) ppm.

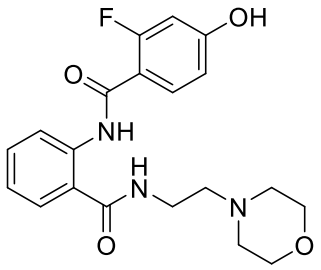
MS(ESI)$^-$: 373.16 $[M-H]^-$

m.p.: -

Synthesis of 2-fluoro-4-hydroxy-*N*-(2-((2-morpholinoethyl)carbamoyl)phenyl)benzamide (127)

Chemical Formula: $C_{20}H_{22}FN_3O_4$

Molecular Weight: 387.41



Procedure: A solution was prepared by adding the starting material (1 equivalent), BBr_3 (3 equivalent), anhydrous dichloromethane (2 mL / mmol) at -78 °C. The reaction mixture was stirred for 16 hours and left to reach 25 °C.

The reaction mixture was quenched with iced water, extracted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered and evaporated to give the desired product.

State: white powder

Yield: 38%

^{1}H-NMR ($CDCl_3$): δ 2.49 (s, 4H), 2.61 (d, J = 5.9 Hz, 2H), 3.50 (dd, J = 11.2, 5.4 Hz, 2H), 3.68 (dd, J = 8.7, 13.2 Hz, 4H), 6.51 – 6.61 (m, 1H), 6.62 (ddd, J = 2.1, 10.6, 14.8 Hz, 2H), 7.00 – 7.14 (m, 2H), 7.32 – 7.53 (m, 2H), 7.82 (t, J = 8.8 Hz, 1H), 8.56 (d, J = 8.0 Hz, 1H), 11.27 (d, J = 8.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.11 (CH_2, C-aliphatic), 53.21 (CH_2, C-aliphatic), 56.41 (CH_2, C-aliphatic), 66.83 (CH_2, C-aliphatic), 76.77 (CH_2, C-aliphatic), 103.41 (CH, C-aromatic), 112.46 (CH, C-aromatic), 122.21 (CH, C-aromatic), 122.86 (C, C-aromatic), 123.44 (CH, C-aromatic), 126.72 (CH, C-aromatic), 132.91 (CH, C-aromatic), 132.94 (CH, C-aromatic), 138.86 (C, C-aromatic), 160.45 (C, C-aromatic), 161.36 (C, C-aromatic), 162.44 (C, C-aromatic), 169.38 (C, C-aromatic) ppm.

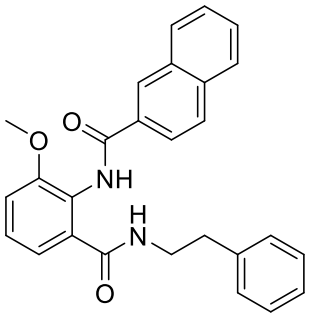
^{19}F-NMR ($CDCl_3$): δ –109.64 ppm.

MS(ESI)$^-$: 386.24 $[M+H]^-$

m.p.: 138 – 104 °C

Synthesis of N-(2-methoxy-6-(phenylcarbamoyl)phenyl)-2-naphthamide (128)

Chemical Molecular Formula: $C_{27}H_{24}N_2O_3$
Molecular Weight: 424.49



Procedure: 2

Yield: 50 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.86-2.89 (m, 2H), 3.62-3.66 (m, 2H), 3.899 (s, 3H), 6.40-6.43 (m, 1H), 7.04-7.06 (m, 1H), 7.08-7.10 (m, 1H), 7.195 (t, 3H), 7.25-7.28 (m, 3H), 7.57-7.63 (m, 2H), 7.92-8.04 (4H, m), 8.51 (d, 1H), 9.03 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.46 (CH_2, C-aliphatic), 41.005 (CH_2, C-aliphatic), 56.20 (-CH_3, C-aliphatic), 113.72 (CH, C-aromatic), 119.23 (CH, C-aromatic), 124.07 (CH, C-aromatic), 124.49 (C, C-aromatic), 126.51 (CH, C-aromatic), 126.78 (CH, C-aromatic), 127.07 (CH, C-aromatic), 127.79 (CH, C-aromatic), 127.89 (CH, C-aromatic), 128.42 (CH, C-aromatic), 128.59 (CH, C-aromatic) 128.62 (CH, C-aromatic), 128.76 (CH, C-aromatic), 129.21 (CH, C-aromatic), 131.34 (C, C-aromatic), 132.67 (C, C-aromatic), 135.07 (C, C-aromatic), 138.74 (C, C-aromatic), 154.18 (C, C-aromatic), 165.66 (C, C-aromatic), 166.57 (C, C-aromatic) ppm.

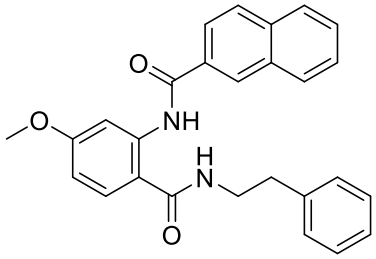
MS(ESI)$^+$: 425.2 $[M+H]^+$

m.p: 188-190 °C

Synthesis of N-(5-methoxy-2-(phenethylcarbamoyl)phenyl)-2-naphthamide (129)

Molecular Formula**:** $C_{27}H_{24}N_2O_3$

Molecular Weight: 424.49



Procedure: 2

Yield: 29 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.99 (t, J = 6.8 Hz, 2H), 3.78 (dd, J = 1.7, 6.8 Hz, 2H), 6.18 (s, 1H), 3.95 (s, 3H), 6.63 (dd, J = 2.6, 8.8 Hz, 1H), 7.25 – 7.32 (m, 7H), 7.34 – 7.41 (m, 2H), 7.56 – 7.66 (m, 2H), 7.93 (d, J = 7.7 Hz, 1H), 8.01 (d, J = 8.6 Hz, 1H), 8.07 (d, J = 7.7 Hz, 1H), 8.14 (dd, J = 1.8, 8.6 Hz, 1H), 8.58 – 8.70 (m, 2H), 12.77 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.65 (CH_2, C-aliphatic), 40.99 (CH_2, C-aliphatic), 55.58 (CH_3, C-aliphatic), 105.22 (CH, C-aromatic), 110.10 (CH, C-aromatic), 112.34 (C, C-aromatic), 123.66 (CH, C-aromatic), 126.64 (CH, C-aromatic), 126.77 (CH, C-aromatic), 127.62 (CH, C-aromatic), 127.72 (CH, C-aromatic), 127.83 (CH, C-aromatic), 127.85 (CH, C-aromatic), 128.52 (CH, C-aromatic), 128.66 (CH, C-aromatic), 128.82 (CH, C-aromatic) 129.47 (CH, C-aromatic), 132.13 (C, C-aromatic), 132.82 (C, C-aromatic), 135.01 (C, C-aromatic), 138.66 (C, C-aromatic), 142.54 (C, C-aromatic), 163.02 (C, C-aromatic), 135.92 (C, C-aromatic), 169.05 (C, C-aromatic) ppm.

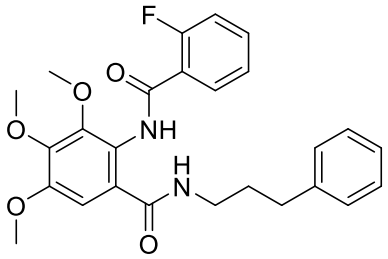
m.p: 119-121 °C (from ethanol/water)

MS (ESI)$^+$: 448.2 $[M+Na]^+$

Synthesis of 2(2-fluorobenzamido)-3,4,5-trimethoxy-N-(3-phenylpropyl)benzamide (130)

Molecular Formula: $C_{26}H_{27}FN_2O_5$

Molecular Weight: 466.50



Procedure: 2

Yield: 78%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.73 (dt, J = 7.5, 14.8 Hz, 2H), 2.60 – 2.44 (m, 2H), 3.31 (dd, J = 7.0, 13.0 Hz, 2H), 3.82 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 6.56 (s, 1H), 6.84 (s, 1H), 7.03 – 6.97 (m, 2H), 7.05 – 7.13 (m, 2H), 7.11 – 7.18 (m, 3H), 7.39 – 7.48 (m, 1H), 8.02 (td, J = 1.8, 7.8 Hz, 1H), 8.37 (d, J = 13.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 31.06 (CH_2, C-aliphatic), 33.14 (CH_2, C-aliphatic), 39.54 (CH_2, C-aliphatic), 56.29 (CH_3, C-aliphatic), 61.03 (CH_3, C-aliphatic), 61.23 (CH_3, C-aliphatic), 106.76 (CH, C-aromatic), 116.27 (CH, C-aromatic), 116.47 (CH, C-aromatic), 120.59 (C, C-aromatic), 124.95 (CH, C-aromatic), 125.87 (CH, C-aromatic), 128.30 (CH, C-aromatic), 128.35 (CH, C-aromatic), 130.25 (C, C-aromatic), 132.24 (CH, C-aromatic), 134.03 (CH, C-aromatic) 134.10 (CH, C-aromatic), 141.37 (C, C-aromatic), 144.07 (C, C-aromatic), 149.13 (C, C-aromatic), 152.73 (C, C-aromatic), 159.87 (C, C-aromatic), 161.85 (C, C-aromatic), 166.92 (C, C-aromatic), 167.53 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.27 ppm.

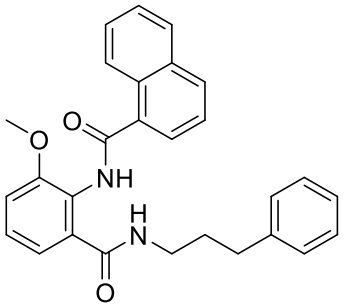
m.p.: 145-147 °C

MS (ESI)$^{+}$: 489.2 $[M+Na]^{+}$

Synthesis of N-(2-methoxy-6-(3-phenylpropylcarbamoyl)phenyl)-1-naphthamide (131)

Molecular Formula: $C_{26}H_{26}N_2O_3$

Molecular Weight: 438.51



Procedure: 2

Yield: 69 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.82 – 2.00 (m, 2H), 2.59 – 2.81 (m, 2H), 3.47 (dd, J = 7.0, 13.1 Hz, 2H), 3.93 (s, 3H), 6.48 (s, 1H), 7.04 – 7.12 (m, 2H), 7.17 (dd, J = 7.1, 18.8 Hz, 3H), 7.24 (t, J = 7.2 Hz, 2H), 7.31 (t, J = 8.1 Hz, 1H), 7.47 – 7.53 (m, 1H), 7.55 – 7.59 (m, 2H), 7.87 (d, J = 6.9 Hz, 1H), 7.90– 7.96 (m, 1H), 7.99 (d, J = 8.2 Hz, 1H), 8.21 (s, 1H), 8.55 (dd, J = 3.6, 6.1 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 31.13 (CH_2, C-aliphatic), 33.35 (CH_2, C-aliphatic), 39.73 (CH_2, C-aliphatic), 56.13 (CH_3, C-aliphatic), 113.28 (CH, C-aromatic), 119.51 (CH, C-aromatic), 123.54 (C, C-aromatic), 124.83 (CH, C-aromatic), 125.64 (CH, C-aromatic), 125.91 (CH, C-aromatic), 125.97 (CH, C-aromatic), 126.44 (CH, C-aromatic), 127.25 (CH, C-aromatic), 127.52 (CH, C-aromatic), 128.31 (CH, C-aromatic) 128.40 (CH, C-aromatic), 128.42 (CH, C-aromatic), 130.45 (C, C-aromatic), 131.21 (CH, C-aromatic), 133.73 (C, C-aromatic), 133.79 (C, C-aromatic), 134.30 (C, C-aromatic), 141.42 (C, C-aromatic), 154.23 (C, C-aromatic), 168.34 (C, C-atomatic), 168.96 (C, C-aromatic) ppm.

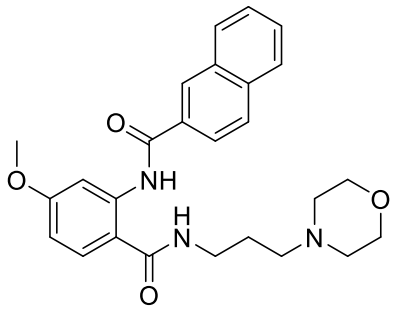
MS (ESI)$^+$: 461.2 $[M+Na]^+$

m.p: (from ethyl acetate/ *n*-hexane) 150-152 °C

Synthesis of N-(5-methoxy-2-(3-morpholinepropylcarbamoyl)phenyl)-2-naphthamide (132)

Molecular Formula: $C_{26}H_{29}N_3O_4$

Molecular Weight: 447.52



Procedure: 2

Yield: 44%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.74 (dt, J = 5.9, 11.6, 2H), 2.46 (s, 4H), 2.55 – 2.50 (m, 2H), 3.55 (dd, J = 5.9, 10.9 Hz, 2H), 3.61 – 3.73 (m, 4H), 3.87 (3H, s), 6.60 (dd, J = 2.6, 8.8 Hz, 1H), 7.41 – 7.53 (m, 1H), 7.82 (d, J = 7.9 Hz, 2H), 7.89 (d, J = 8.6 Hz, 1H), 7.96 (d, J = 7.8 Hz, 2H), 8.05 (dd, J = 1.8, 8.6 Hz, 2H), 8.39 (s, 1H), 8.49 – 8.62 (m, 1H), 12.87 – 12.94 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.52 (CH_2, C-aliphatic), 41.02 (CH_2, C-aliphatic), 53.91 (CH_2, C-aliphatic), 55.59 (CH_3, C-aliphatic), 59.16 (CH_2, C-aliphatic), 66.94 (CH_2, C-aliphatic), 105.16 (CH, C-aromatic), 109.86 (CH, C-aromatic), 112.44 (C, C-aromatic), 123.66 (CH, C-aromatic), 126.60 (CH, C-aromatic), 127.71 (CH, C-aromatic), 127.78 (CH, C-aromatic), 128.15 (CH, C-aromatic), 128.54 (CH, C-aromatic), 128.63 (CH, C-aromatic), 129.46 (CH, C-aromatic), 132.21 (C, C-aromatic), 132.83 (C, C-aromatic), 134.98 (C, C-aromatic), 142.61 (C, C-aromatic), 162.93 (C, C-aromatic), 165.94 (C, C-aromatic), 169.10 (C, C-aromatic) ppm.

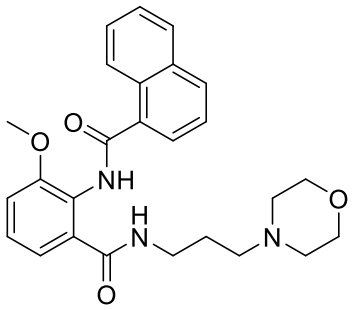
MS (ESI)$^-$: 446.2 $[M-H]^-$

m.p: (from ethanol/water) 131-133 °C

Synthesis of N-(2-methoxy-6-(3-morpholinopropylcarbamoyl)phenyl)-1-naphtamide (133)

Molecular Formula: $C_{26}H_{29}N_3O_4$

Molecular Weight: 447.53



Procedure: 2

Yield: 24%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.68 – 1.93 (m, 2H), 2.45 (s, 4H), 2.52 (t, J = 6.0 Hz, 2H), 3.53 (dd, J = 5.8, 11.6 Hz, 2H), 3.63 (s, 4H), 3.95 (s, 3H), 7.01 – 7.24 (m, 2H), 7.48 – 7.61 (m, 3H), 7.85 (s, 1H), 7.89 – 7.94 (m, 2H), 7.98 (d, J = 8.2 Hz, 1H), 8.59 (d, J = 7.3 Hz, 2H), 11.58 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.59 (CH_2, C-aliphatic), 39.98 (CH_2, C-aliphatic), 53.61 (CH_2, C-aliphatic), 56.18 (CH_3, C-aliphatic), 57.96 (CH_2, C-aliphatic), 66.85 (CH_2, C-aliphatic), 113.55 (CH, C-aromatic), 119.17 (CH, C-aromatic), 124.86 (CH, C-aromatic), 125.78 (CH, C-aromatic), 125.96 (CH, C-aromatic), 126.38 (CH, C-aromatic), 126.93 (CH, C-aromatic), 127.16 (CH, C-aromatic), 127.20 (C, C-aromatic), 128.28 (CH, C-aromatic), 130.55 (C, C-aromatic), 131.09 (CH, C-aromatic), 133.09 (C, C-aromatic), 133.17 (C, C-aromatic), 133.80 (C, C-aromatic), 133.95 (C, C-aromatic), 154.22 (C, C-aromatic), 168.37 (C, C-aromatic) ppm.

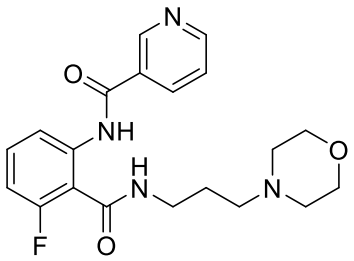
MS (ESI)$^-$: 446.2 $[M-H]^-$

m.p.: (from ethanol/water) 130-132 °C

Synthesis of *N*-(2-((3-morpholinopropyl)carbamoyl)phenyl)nicotinamide (134)

Chemical Molecular Formula: $C_{20}H_{24}N_4O_3$

Molecular Weight: 368.18



Procedure: 2

State: white powder

Yield: 79 %

^{1}H-NMR ($CDCl_3$): δ 1.75 (dt, J = 5.8, 11.6 Hz, 2H), 2.46 (s, 4H), 2.52 – 2.56 (m, 2H), 3.52 (dd, J = 5.9, 10.6 Hz, 2H), 3.64 (t, J = 4.5 Hz, 4H), 7.10 (td, J = 1.1, 7.8 Hz, 1H), 7.37 (ddd, J = 0.7, 4.8, 7.9 Hz, 1H), 7.47 – 7.51 (m, 1H), 7.54 – 7.58 (m, 1H), 8.25 (ddd, J = 1.7, 2.2, 8.0 Hz, 1H), 8.67 (s, 1H), 8.70 (dd, J = 1.6, 4.8 Hz, 1H), 8.76 (dd, J = 1.0, 8.4 Hz, 1H), 9.24 (d, J = 1.8 Hz, 1H), 12.62 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.41 (CH_2, C-aliphatic), 33.58 (CH_2, C-aliphatic), 39.82 (CH_2, C-aliphatic), 57.62 (CH_2, C-aliphatic), 60.10 (CH_2, C-aliphatic), 124.46 (CH, C-aromatic), 128.52 (CH, C-aromatic), 128.73 (CH, C-aromatic), 130.07 (C,C-aromatic), 130.47 (CH, C-aromatic), 132.85 (CH, C-aromatic), 134.86 (CH, C-aromatic), 139.73 (C, C-aromatic), 141.21 (C, C-aromatic), 149.20 (CH, C-aromatic), 152.48 (CH, C-aromatic), 163.75 (C, C-aromatic), 169.01 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -110.50 ppm.

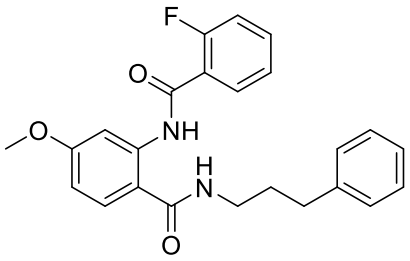
MS (ESI)$^-$: 385.2 $[M-H]^-$

m.p.: (from acetone/*n*-hexane) 120-122 °C

Synthesis of 2-(2-fluorobenzamido)-4-methoxy-*N*-(3-phenylpropyl)benzamide (135)

Chemical Formula: $C_{24}H_{23}FN_2O_3$

Molecular Weight: 406.46



Procedure: 2

State: white powder

Yield: 49 %

^{1}H-NMR ($CDCl_3$): δ 1.83 – 1.94 (m, 2H), 2.58 – 2.69 (m, 2H), 3.40 (dd, J = 6.9, 12.8 Hz, 2H), 3.81 (d, J = 8.1 Hz, 3H), 6.06 (s, 1H), 6.50 (dd, J = 2.6, 8.7 Hz, 1H), 7.07 – 7.14 (m, 4H), 7.16 – 7.24 (m, 4H), 7.36 – 7.48 (m, 1H), 7.92 (td, J = 1.8, 7.7 Hz, 1H), 8.39 (d, J = 2.6 Hz, 1H), 12.11 (d, J = 6.0 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.95 (CH_2, C-aliphatic), 33.60 (CH_2, C-aliphatic), 39.75 (CH_2, C-aliphatic), 55.55 (CH_3, C-aliphatic), 106.11 (CH, C-aromatic), 110.07 (CH, C-aromatic), 113.58 (C, C-aromatic), 116.56 (CH, C-aromatic), 116.74 (CH, C-aromatic), 124.62 (CH, C-aromatic), 126.15 (CH, C-aromatic), 127.83 (CH, C-aromatic), 128.38 (CH, C-aromatic), 128.65 (CH, C-aromatic), 131.27 (CH, C-aromatic), 131.60 (C, C-aromatic), 133.39 (C, C-aromatic), 141.42 (C, C-aromatic), 161.33 (C, C-aromatic), 162.65 (C, C-aromatic), 168.54 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.88 ppm.

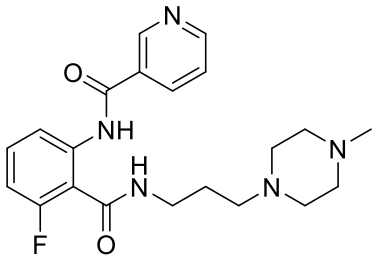
MS (ESI)$^+$: 429.1 $[M+Na]^+$

m.p.: 80 - 82°C

Synthesis of N-(3-fluoro-2-(3-(4-methylpiperazin-1-yl)propylcarbamoyl)phenyl)nicotinamide (136)

Molecular Formula: $C_{21}H_{26}FN_5O_2$

Molecular Weight: 399.46



Yield: 24 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.60-1.68 (m, 2H), 1.72 – 1.83 (m, 2H), 2.19 (s, 5H), 2.32 (s, 3H), 2.43 – 2.55 (m, 3H), 3.53 (dd, J = 5.0, 11.0 Hz, 2H), 6.83 (ddd, J = 1.0, 8.3, 11.6 Hz, 1H), 7.32 – 7.42 (m, 2H), 8.23 (ddd, J = 1.7, 2.3, 8.0 Hz, 1H), 8.43 (s, 1H), 8.52 (t, J = 23.0 Hz, 1H), 8.71 (dd, J = 1.6, 4.8 Hz, 1H), 9.22 (d, J = 1.7 Hz, 1H), 12.54 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.28 (CH_2, C-aliphatic), 40.47 (CH_2, C-aliphatic), 46.01 (CH_3, C-aliphatic), 53.27 (CH_2, C-aliphatic), 54.87 (CH_2, C-aliphatic), 57.68 (CH_2, C-aliphatic), 110.75 (CH, C-aromatic), 117.42 (CH, C-aromatic), 123.48 (CH, C-aromatic), 130.29 (C, C-aromatic), 132.86 (CH, C-aromatic), 134.89 (CH, C-aromatic), 141.22 (C, C-aromatic), 149.26 (CH, C-aromatic), 152.60 (CH, C-aromatic), 159.40 (C, C-aromatic), 161.36 (C, C-aromatic), 163.80 (C, C-aromatic), 164.91 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -110.05 ppm.

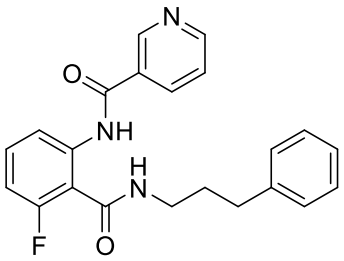
MS (ESI)$^-$: 398.2 $[M-H]^-$

m.p.: 72-74 °C

Synthesis of N-(3-fluoro-2-(3-phenylpropylcarbamoyl)phenyl)nicotinamide (137)

Molecular Formula: $C_{22}H_{20}FN_3O_2$

Molecular Weight: 377.41



Procedure: 2

Yield: 67%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.91 – 2.09 (m, 2H), 2.71 – 2.80 (m, 2H), 3.55 (td, J = 1.4, 7.2 Hz, 2H), 6.93 (ddd, J = 1.0, 8.3, 12.6 Hz, 2H), 7.20 – 7.27 (m, 1H), 7.27 – 7.35 (m, 4H), 7.43 – 7.53 (m, 2H), 8.27 – 8.39 (m, 1H), 8.71 (d, J = 8.5 Hz, 1H), 8.82 (s, 1H), 9.33 (s, 1H), 12.93 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.72 (CH_2, C-aliphatic), 33.21 (CH_2, C-aliphatic), 39.63 (CH_2, C-aliphatic), 110.60 (CH, C-aromatic), 110.80 (CH, C-aromatic), 117.58 (CH, C-aromatic), 123.51 (CH, C-aromatic), 126.17 (CH, C-aromatic), 128.36 (CH, C-aromatic), 128.56 (CH, C-aromatic), 133.21 (CH, C-aromatic), 133.31 (CH, C-aromatic), 134.93 (CH, C-aromatic), 140.97 (C, C-aromatic), 141.83 (C, C-aromatic), 149.24 (CH, C-aromatic), 152.61 (CH, C-aromatic), 159.96 (C, C-aromatic), 161.91 (C, C-aromatic), 163.90 (C, C-aromatic), 165.62 (C, C-aromatic), 210.12 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -110.84 ppm.

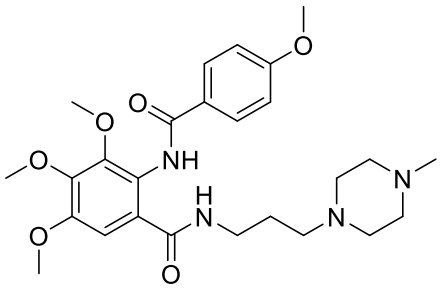
MS (ESI)$^-$: 376.1 $[M-H]^-$

m.p: (from ethanol/water) 79-81 °C

Synthesis of 3, 4, 5-trimethoxy-2-(4-methoxybenzamido)-N-(3-(4-methylpiperazin)-1-yl)propyl)benzamide (138)

Molecular Formula**:** $C_{26}H_{36}N_4O_6$

Molecular Weight: 500.58



Yield: 30 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.48 – 1.73 (m, 2H), 2.18 (s, 4H), 2.35 (t, J = 6.5 Hz, 9H), 3.34 (dd, J = 6.2, 11.9 Hz, 2H), 3.80 (s, 3H), 3.82 (s, 3H), 3.85 (s, 3H), 3.85 (s, 3H), 6.79 (s, 1H), 6.85 – 6.96 (m, 2H), 7.54 (s, 1H), 7.83 – 7.92 (m, 2H), 8.63 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 25.22 (CH_2, C-aliphatic), 39.24 (CH_2, C-aliphatic), 45.70 (CH_3, C-aliphatic), 52.71 (CH_2, C-aliphatic), 54.65 (CH_2, C-aliphatic), 55.49 (CH_3, C-aliphatic), 56.62 (CH_2, C-aliphatic), 56.70 (CH_3, C-aliphatic), 61.01 (CH_3, C-aliphatic), 61.03 (CH_3, C-aliphatic), 106.79 (CH, C-aromatic), 113.95 (CH, C-aromatic), 123.01 (C, C-aromatic), 126.35 (C, C-aromatic), 127.81 (C, C-aromatic), 129.51 (CH, C-aromatic), 144.73 (C, C-aromatic), 149.41 (C, C-aromatic), 151.82 (C, C-aromatic), 162.68 (C, C-aromatic), 166.67 (C, C-aromatic), 168.05 (C, C-aromatic) ppm.

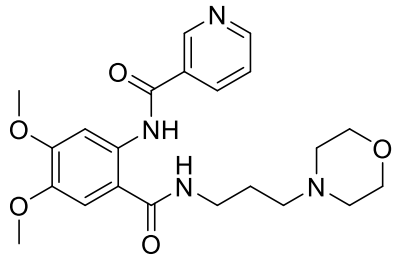
MS (ESI)$^-$: 499.3 $[M-H]^-$

m.p.: 94-96° C

Synthesis of N-(4, 5-dimethoxy-2-(3-morpholinopropylcarbamoyl)phenyl)nicotinamide (139)

Molecular Formula: $C_{22}H_{28}N_4O_5$

Molecular Weight: 428.48



Procedure: 2

Yield: 74 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.81 (d, J = 16.7 Hz, 2H), 1.83 – 1.91 (m, 2H), 2.53 (s, 3H), 2.58 (dd, J = 11.0, 17.1 Hz, 2H), 3.60 (dd, J = 5.6, 11.4 Hz, 2H), 3.73 (d, J = 4.0 Hz, 3H), 3.95 (s, 3H), 4.04 (d, J = 7.5 Hz, 2H), 7.10 (s, 1H), 7.47 – 7.64 (m, 1H), 7.98 (s, 1H), 8.24 – 8.43 (m, 1H), 8.63 (d, J = 22.8 Hz, 1H), 8.79 (dd, J = 1.4, 4.8 Hz, 1H), 9.32 (d, J = 1.8 Hz, 1H), 12.85 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.17 (CH_2, C-aliphatic), 40.30 (CH_2, C-aliphatic), 53.82 (CH_2, C-aliphatic), 56.12 (CH_3, C-aliphatic), 57.56 (CH_3, C-aliphatic), 58.34 (CH_2, C-aliphatic), 66.74 (CH_2, C-aliphatic), 104.93 (CH, C-aromatic), 111.67 (CH, C-aromatic), 111.77 (C, C-aromatic), 123.49 (CH, C-aromatic), 130.57 (C, C-aromatic), 134.78 (CH, C-aromatic), 136.03 (C, C-aromatic), 144.47 (C, C-aromatic), 149.14 (CH, C-aromatic), 152.37 (CH, C-aromatic), 153.09 (C, C-aromatic), 163.66 (C, C-aromatic), 168.95 (C, C-aromatic) ppm.

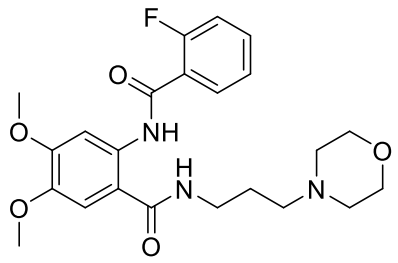
MS (ESI)$^-$: 427.3 $[M-H]^-$

m.p: (from ethyl acetate/*n*-hexane) 69-71 °C

Synthesis of 2-(2-fluorobenzamido)-4,5-dimethoxy-N-(3-morpholinopropyl)benzamide (140)

Molecular Formula: $C_{23}H_{28}FN_2O_5$

Molecular Weight: 445.49



Procedure: 2

Yield: 46%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.68 – 1.71 (m, 2H). 2.43 (s, 3H), 2.46 (bs, 4H), 3.47 (dt, J = 15.4, 30.9 Hz, 2H), 3.51 – 3.71 (m, 3H), 3.85 (s, 3H), 3.93 (s, 3H), 6.98 (s, 1H), 7.12 (ddd, J = 0.9, 8.3, 11.2 Hz, 2H), 7.42 (dddd, J = 1.8, 5.1, 7.1, 8.3 Hz, 1H), 7.67 (s, 1H), 7.95 (td, J = 1.8, 7.7 Hz, 1H), 8.47 (s, 1H), 12.01 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.30 (CH_2, C-aliphatic), 40.11 (CH_2, C-aliphatic), 53.76 (CH_2, C-aliphatic), 56.09 (CH_3, C-aliphatic), 57.34 (CH_3, C-aliphatic), 58.24 (CH_2, C-aliphatic), 66.72 (CH_2, C-aliphatic), 105.82 (CH, C-aromatic), 111.34 (CH, C-aromatic), 116.50 (CH, C-aromatic), 116.69 (CH, C-aromatic), 124.59 (CH, C-aromatic), 124.62 (CH, C-aromatic), 131.26 (CH, C-aromatic), 133.28 (C, C-aromatic), 135.04 (C, C-aromatic), 144.51 (C, C-aromatic), 152.48 (C, C-aromatic), 159.03 (C, C-aromatic), 161.27 (C, C-aromatic), 162.99 (C, C-aromatic), 168.55 (C, C-aromatic)

^{19}F-NMR ($CDCl_3$): δ -112.59 ppm.

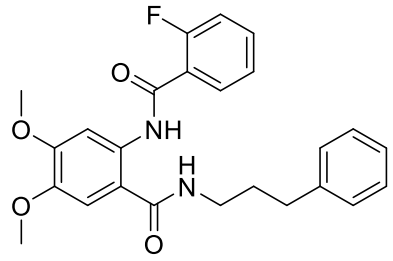
MS (ESI)$^-$: 444.2 $[M-H]^-$

m.p.: 75-77 °C

Synthesis of 2-(2-fluorobenzamido)-4,5-dimethoxy-N-(3-phenylpropyl)benzamide (141)

Molecular Formula: $C_{25}H_{25}FN_2O_4$

Molecular Weight: 436.48



Procedure: 2

Yield: 58 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.91 – 2.09 (m, 2H), 2.74 (d, J = 7.6 Hz, 2H), 3.44 – 3.55 (m, 2H), 3.90 (s, 3H), 4.04 (s, 3H), 6.02 (s, 1H), 6.81 (s, 1H), 7.13 – 7.26 (m, 2H), 7.22 – 7.36 (m, 5H), 7.44 – 7.57 (m, 1H), 8.05 (td, J = 1.8, 7.7 Hz, 1H), 8.54 (s, 1H), 11.92 (d, J = 6.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 31.02 (CH_2, C-aliphatic), 33.49 (CH_2, C-aliphatic), 39. 78 (CH_2, C-aliphatic), 56.13 (CH_3, C-aliphatic), 56.62 (CH_3, C-aliphatic), 105.82 (CH, C-aromatic), 109.52 (CH, C-aromatic), 113.37 (C, C-aromatic), 116.51 (CH, C-aromatic), 116.69 (CH, C-aromatic), 124.61 (CH, C-aromatic), 124.64 (CH, C-aromatic), 126.17 (CH, C-aromatic), 128.57 (CH, C-aromatic), 131.29 (CH, C-aromatic), 131.30 (C, C-aromatic), 133.33 (C, C-aromatic), 134.63 (C, C-aromatic), 141.29 (C, C-aromatic), 144.54 (C, C-aromatic), 152.08 (C, C-aromatic), 161.39 (C, C-aromatic), 168.46 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.59 ppm.

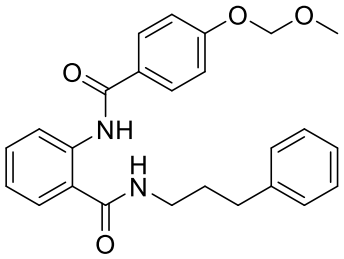
MS (ESI)$^{-}$: 435.2 $[M-H]^-$

m**.p.:** (from ethyl acetate/*n*-hexane) 89-91 °C

Synthesis of 2-(4-(methoxymethoxy)benzamido)-*N*-(3-phenylpropyl)benzamide (142)

Chemical Formula: $C_{25}H_{26}N_2O_4$

Molecular Weight: 418.49



Procedure: To a stirred solution of intermediate **299** (1 equivalent) in dichloromethane (1.3 mmol/mL), EDCI (1.2 equivalents) and HOBt (1.2 equivalents). The reaction mixture was stirred at room temperature for 20 minutes. Compound **300** (1.5 equivalents) was added. Reaction mixture was stirred for 16 hours. Reaction mixture was diluted with water, extracted with ethyl acetate, washed with brine dried over $MgSO_4$, filtered and evaporated. Crude was purified by silica flash column chromatography, using dichloromethane : methanol as eluent.

Yield: 26%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.79 – 1.93 (m, 2H), 2.59 (d, J = 7.6 Hz, 2H), 3.33 – 3.37 (m, 2H), 3.37 (s, 3H), 5.11 (s, 2H), 6.79 (ddd, J = 3.0, 9.04, 15.2 Hz, 4H), 6.99 – 7.31 (m, 6H), 7.79 – 7.90 (m, 2H), 8.46 – 8.60 (m, 2H), 11.92 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.83 (CH_2, C-aliphatic), 33.51 (CH_2, C-aliphatic), 39.84 (CH_2, C-aliphatic), 50.52 (CH_3, C-aliphatic), 94.11 (CH_2, C-aliphatic), 116.13 (CH, C-aromatic), 120.76 (C, C-aromatic), 121.31 (CH, C-aromatic), 122.63 (CH, C-aromatic), 125.83 (CH, C-aromatic), 126.99 (CH, C-aromatic), 128.22 (C, C-aromatic), 128.41 (CH, C-aromatic), 128.58 (CH, C-aromatic), 129.24 (CH, C-aromatic), 132.16 (CH, C-aromatic), 139.64 (CH, C-aromatic), 141.41 (C, C-aromatic), 160.19 (C, C-aromatic), 161.21 (C, C-aromatic), 165.34 (C, C-aromatic), 169.33 (C, C-aromatic) ppm.

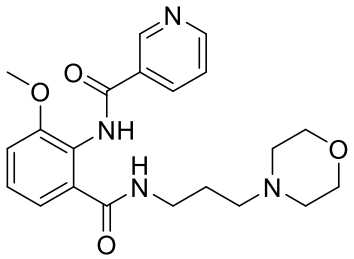
MS (ESI)$^-$: 417.3 $[M-H]^-$

m.p.: (from ethyl acetate/*n*-hexane) 125 - 127 °C

Synthesis of *N*-(2-methoxy-6-((3-morpholinopropyl)carbamoyl)phenyl)nicotinamide (143)

Chemical Formula: $C_{21}H_{26}N_4O_4$

Molecular Weight: 398.46



Procedure: 2

Yield: 51 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.70 (dd, J = 5.9, 11.9 Hz, 2H), 2.44 (s, 4H), 2.49 (s, 2H), 3.40 (dd, J = 5.7, 11.5 Hz, 2H), 3.61 (s, 4H), 3.84 (s, 3H), 7.04 (ddd, J = 14.2, 15.2, 29.9 Hz, 2H), 7.18 – 7.24 (m, 1H), 7.34 (ddd, J = 0.7, 4.9, 7.9 Hz, 1H), 8.03 (s, 1H), 8.21 – 8.14 (m, 1H), 8.69 (dd, J = 1.6, 4.8 Hz, 1H), 9.14 (d, J = 1.7 Hz, 1H), 9.55 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.91 (CH_2, C-aliphatic), 40.17 (CH_2, C-aliphatic), 53.61 (CH_2, C-aliphatic), 56.26 (CH_3, C-aliphatic), 58.20 (CH_2, C-aliphatic), 66.67 (CH_2, C-aliphatic), 114.31 (CH, C-aromatic), 118.90 (CH, C-aromatic), 123.42 (CH, C-aromatic), 125.38 (C, C-aromatic), 126.68 (CH, C-aromatic), 130.14 (C, C-aromatic), 130.89 (C, C-aromatic), 135.36 (CH, C-aromatic), 149.19 (CH, C-aromatic), 152.47 (CH, C-aromatic), 154.33 (C, C-aromatic), 163.94 (C, C-aromatic), 168.40 (C, C-aromatic) ppm.

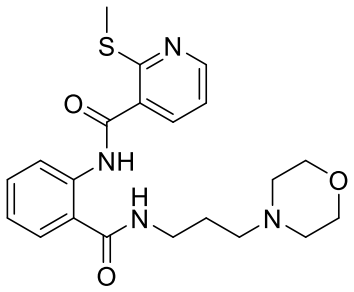
MS (ESI)$^-$: 397.3 $[M-H]^-$

m.p.: (from ethyl acetate/*n*-hexane) 125 - 127 °C

Synthesis of 2-(methylthio)-*N*-(2-((3-morpholinopropyl)carbamoyl)phenyl)nicotinamide (144)

Molecular Formula: $C_{21}H_{26}N_4O_3S$

Molecular Weight: 414.52



Procedure: 2

Yield: 68 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.73 (dt, J = 5.9, 11.4 Hz, 2H), 2.46 (s, 3H), 2.48 (s, 4H), 2.51 – 2.55 (m, 2H), 3.47 (dd, J = 5.8, 10.7 Hz, 2H), 3.64 (d, J = 4.1 Hz, 4H), 7.02 (dd, J = 12.0, 16.9 Hz, 1H), 7.04 – 7.14 (m, 1H), 7.43 – 7.52 (m, 1H), 7.52 (d, J = 7.8 Hz, 1H), 7.89 (dd, J = 1.7, 7.7 Hz, 1H), 8.49 (dd, J = 1.7, 4.8 Hz, 1H), 8.61 (s, 1H), 8.71 (dd, J = 0.9, 8.4 Hz, 1H), 12.11 (s, 1H) ppm.

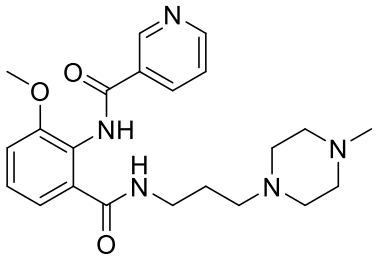
^{13}C-NMR ($CDCl_3$): δ 13.91 (CH_3, C-aliphatic), 23.25 (CH_2, C-aliphatic), 40.96 (CH_2, C-aliphatic), 53.79 (CH_2, C-aliphatic), 58.96 (CH_2, C-aliphatic), 66.76 (CH_2, C-aliphatic), 118.49 (CH, C-aromatic), 120.47 (C, C-aromatic), 121.76 (CH, C-aromatic), 123.02 (CH, C-aromatic), 126.79 (CH, C-aromatic), 129.04 (C, C-aromatic), 132.71 (CH, C-aromatic), 134.84 (CH, C-aromatic), 139.75 (C, C-aromatic), 150.69 (CH, C-aromatic), 160.57 (C, C-aromatic), 165.19 (C, C-aromatic), 169.02 (C, C-aromatic) ppm.

MS (ESI)$^-$: 413.17 $[M-H]^-$

m.p.: 128 – 130 °C

Synthesis of *N*-(2-methoxy-6-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)nicotinamide (145)

Chemical Formula: $C_{22}H_{29}N_5O_3$
Molecular Weight: 411.51



Procedure: 2

Yield: 32 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.68 (dt, J = 7.0, 11.8 Hz, 4H), 2.19 (s, 3H), 2.26 – 2.44 (m,4H), 2.45 – 2.53 (m, 4H), 3.39 (dd, J = 5.7, 11.0 Hz, 2H), 3.85 (s, 3H), 7.02 – 7.13 (m, 2H), 7.17 – 7.22 (m, 1H), 7.34 (ddd, J = 0.7, 4.9, 7.9 Hz, 1H), 8.15 – 8.26 (m, 1H), 8.34 (s, 1H), 8.69 (dd, J = 1.6, 4.8 Hz, 1H), 9.15 (d, J = 1.6 Hz, 1H), 9.80 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.95 (CH_2, C-aliphatic), 40.78 (CH_2, C-aliphatic), 45.97 (CH_3, C-aliphatic), 53.15 (CH_2, C-aliphatic), 54.99 (CH_2, C-aliphatic), 56.29 (CH_3, C-aliphatic), 58.09 (CH_2, C-aliphatic), 114.49 (CH, C-aromatic), 119.26 (CH, C-aromatic), 123.40 (CH, C-aromatic), 125.83 (C, C-aromatic), 126.41 (CH, C-aromatic), 130.17 (C, C-aromatic), 130.36 (CH, C-aromatic), 135.38 (CH, C-aromatic), 149.25 (CH, C-aromatic), 152.43 (C, C-aromatic), 154.40 (C, C-aromatic), 163.80 (C, C-aromatic), 168.32 (C, C-aromatic) ppm.

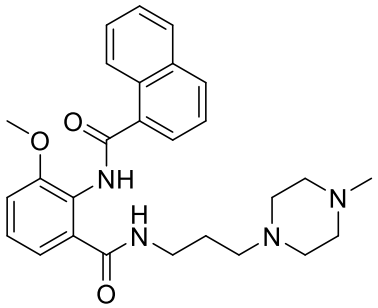
MS (ESI)$^-$: 410.3 $[M-H]^-$

m.p.: 71 – 73 °C

Synthesis of *N*-(2-methoxy-6-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)-1-naphthamide (146)

Chemical Formula: $C_{27}H_{32}N_4O_3$

Molecular Weight: 460.58



Procedure: 2
Yield: 48 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.78 (dt, J = 5.9, 11.9 Hz, 4H), 2.22 (d, J = 30.6 Hz, 4H), 2.33 (d, J = 56.0 Hz, 4H), 2.55 (s, 3H), 3.52 (dd, J = 5.7, 11.3 Hz, 2H), 3.96 (s, 3H), 7.13 (d, J = 8.2 Hz, 1H), 7.19 (d, J = 7.1 Hz, 1H), 7.28 – 7.35 (m, 1H), 7.45 – 7.62 (m, 3H), 7.91 (d, J = 8.0 Hz, 2H), 7.96 (t, J = 11.9 Hz, 1H), 8.12 (s, 1H), 8.60 (d, J = 8.4 Hz, 1H), 8.82 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.60 (CH_2, C-aliphatic), 40.40 (CH_2, C-aliphatic), 46.00 (CH_3, C-aliphatic), 53.15 (CH_2, C-aliphatic), 55.07 (CH_2, C-aliphatic), 56.21 (CH_3, C-aliphatic), 57.74 (CH_2, C-aliphatic), 113.74 (CH, C-aromatic), 119.45 (CH, C-aromatic), 124.80 (C, C-aromatic), 124.86 (CH, C-aromatic), 125.84 (CH, C-aromatic), 125.97 (CH, C-aromatic), 126.33 (CH, C-aromatic), 126.69 (CH, C-aromatic), 127.11 (CH, C-aromatic), 128.25 (CH, C-aromatic), 130.60 (C, C-aromatic), 131.03 (CH, C-aromatic), 132.65 (C, C-aromatic), 133.81 (C, C-aromatic), 133.99 (C, C-aromatic), 154.33 (C, C-aromatic), 167.99 (C, C-aromatic), 168.28 (C, C-aromatic) ppm.

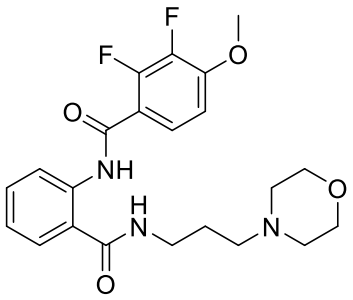
MS (ESI)$^-$: 459.3 $[M-H]^-$

m.p.: 128 – 130 °C

Synthesis of 2,3-difluoro-4-methoxy-*N*-(2-((3-morpholinopropyl)carbamoyl)phenyl)benzamide (147)

Chemical Formula: $C_{22}H_{25}F_2N_3O_4$

Molecular Weight: 433.46



Procedure: 2

Yield: 76 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.62 – 1.79 (m, 2H), 2.31 – 2.48 (m, 4H), 2.50 – 2.54 (m, 2H), 3.50 (dd, J = 5.9, 10.7 Hz, 2H), 3.62 (dd, J = 6.5, 10.8 Hz, 4H), 3.89 (s, 3H), 6.70 – 6.83 (m, 1H), 7.06 (ddd, J = 4.9, 12.4, 18.3 Hz, 1H), 7.39 – 7.55 (m, 2H), 7.63 – 7.77 (m, 1H), 8.41 (s, 1H), 8.64 (dt, J = 11.7, 23.3 Hz, 1H), 11.90 (d, J = 5.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.53 (CH_2, C-aliphatic), 40.97 (CH_2, C-aliphatic), 53.83 (CH_2, C-aliphatic), 56.71 (CH_3, C-aliphatic), 57.80 (CH_2, C-aliphatic), 66.80 (CH_2, C-aliphatic), 108.08 (CH, C-aromatic), 121.77 (C, C-aromatic), 122.38 (CH, C-aromatic), 123.10 (CH, C-aromatic), 125.35 (CH, C-aromatic), 126.79 (CH, C-aromatic), 132.31 (CH, C-aromatic), 139.26 (C, C-aromatic), 148.78 (C, C-aromatic), 148.87 (C, C-aromatic), 150.79 (C, C-aromatic), 151.88 (C, C-aromatic), 161.40 (C, C-aromatic), 168.66 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 136.34, -158.26 ppm.

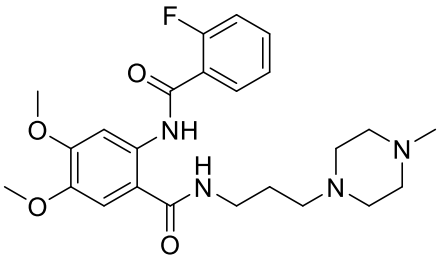
MS (ESI)$^-$: 432.3 $[M-H]^-$

m.p.: 97 – 99 °C

Synthesis of 2-(2-fluorobenzamido)-4,5-dimethoxy-N-(3-(4-methylpiperazin-1-yl)propyl)benzamide (148)

Chemical Formula: $C_{24}H_{31}FN_4O_4$

Molecular Weight: 458.53



Procedure: 2

Yield: 55 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.71 (dt, J = 5.9, 11.8 Hz, 2H), 2.19 (s, 3H), 2.35 (s, 4H), 2.45 – 2.53 (m, 3H), 3.48 (dd, J = 5.5, 11.3 Hz, 2H), 3.87 (s, 3H), 3.93 (s, 3H), 6.99 (s, 1H), 7.12 (dd, J = 1.2, 8.3 Hz, 1H), 7.42 (dd, J = 1.8, 7.1 Hz, 1H), 7.95 (td, J = 1.8, 7.7 Hz, 2H), 8.48 (s, 1H), 12.05 (d, J = 6.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.34 (CH_2, C-aliphatic), 40.75 (CH_2, C-aliphatic), 45.88 (CH_3, C-aliphatic), 53.42 (CH_2, C-aliphatic), 55.03 (CH_3, C-aliphatic), 56.08 (CH_2, C-aliphatic), 57.71 (CH_2, C-aliphatic), 58.27 (CH_3, C-aliphatic), 105.76 (CH, C-aromatic), 112.11 (CH, C-aromatic), 113.63 (C, C-aromatic), 116.52 (CH, C-aromatic), 116.70 (C, C-aromatic), 123.03 (CH, C-aromatic), 123.13 (CH, C-aromatic), 124.57 (CH, C-aromatic), 124.59 (C, C-aromatic), 131.21 (C, C-aromatic), 133.17 (C, C-aromatic), 133.24 (C, C-aromatic), 135.10 (C, C-aromatic), 144.43 (C, C-aromatic), 152.47 (C, C-aromatic), 159.39 (C, C-aromatic), 161.39 (C, C-aromatic), 162.37 (C, C-aromatic), 168.51 (C, C-aromatic) ppm.

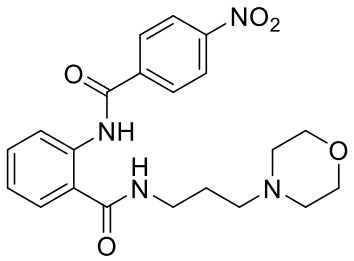
^{19}F-NMR ($CDCl_3$): δ -112.366 ppm.

MS (ESI)$^-$: 457.3 $[M-H]^-$

m.p.: 94 – 96 °C

Synthesis of *N*-(3-morpholinopropyl)-2-(4-nitrobenzamido)benzamide (149)

Chemical Formula: $C_{21}H_{24}N_4O_5$
Molecular Weight: 412.45



Procedure: 2

Yield: 72 %

State: yellow powder

^{1}H-NMR ($CDCl_3$): 1.85 (dt, J = 5.8, 11.5 Hz, 2H), 2.56 (s, 4H), 2.62 – 2.71 (m, 2H), 3.62 (dd, J = 5.8, 10.5 Hz, 2H), 3.75 (t, J = 4.4 Hz, 4H), 7.22 (td, J = 1.1, 7.8 Hz, 1H), 7.57 – 7.63 (m, 2H), 7.67 (dd, J = 1.4, 7.9Hz, 1H), 8.24 (d, J = 2.3 Hz, 1H), 8.33 – 8.53 (m, 2H), 8.86 (dd, J = 0.9, 8.4 Hz, 2H), 12.89 (s, 1H) ppm.

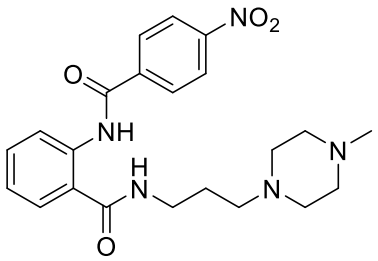
^{13}C-NMR ($CDCl_3$): δ 23.14 (CH_2, C-aliphatic), 41.33 (CH_2, C-aliphatic), 53.88 (CH_2, C-aliphatic), 59.21 (CH_2, C-aliphatic), 66.88 (CH_2, C-aliphatic), 120.13 (C, C-aromatic), 121.51 (CH, C-aromatic), 123.23 (CH, C-aromatic), 123.97 (CH, C-aromatic), 126.89 (CH, C-aromatic), 128.62 (CH, C-aromatic), 132.94 (CH, C-aromatic), 139.88 (C, C-aromatic), 140.56 (C, C-aromatic), 149.76 (C, C-aromatic), 163.31 (C, C-aromatic), 169.08 (C, C-aromatic) ppm.

MS (ESI)$^-$: 411.2 $[M-H]^-$

m.p.: 130 – 132 °C

Synthesis of *N*-(3-(4-methylpiperazin-1-yl)propyl)-2-(4-nitrobenzamido)benzamide (150)
Chemical Formula: $C_{22}H_{27}N_5O_4$

Molecular Weight: 425.49



Procedure: 2

Yield: 76 %

State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 1.66 (s, 2H), 1.84 (dt, J = 5.8, 11.3 Hz, 2H), 2.33 (s, 3H), 2.49 (d, J = 23.2 Hz, 5H), 2.63 – 2.77 (m, 3H), 3.62 (dd, J = 5.7, 10.3 Hz, 2H), 7.20 (td, J = 1.1, 7.8 Hz, 1H), 7.56 – 7.62 (m, 1H), 7.71 (dd, J = 1.4, 7.9 Hz, 1H), 8.20 – 8.31 (m, 2H), 8.34 – 8.46 (m, 2H), 8.86 (dd, J = 0.9, 8.4 Hz, 1H), 9.19 (s, 1H), 12.98 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.22 (CH_2, C-aliphatic), 41.60 (CH_2, C-aliphatic), 46.11 (CH_3, C-aliphatic), 53.38 (CH_2, C-aliphatic), 55.04 (CH_2, C-aliphatic), 58.82 (CH_2, C-aliphatic), 120.09 (C, C-aromatic), 121.36 (CH, C-aromatic), 123.97 (CH, C-aromatic), 127.49 (CH, C-aromatic), 128.63 (CH, C-aromatic), 132.81 (CH, C-aromatic), 139.90 (CH, C-aromatic), 140.61 (C, C-aromatic), 149.74 (C, C-aromatic), 163.33 (C, C-aromatic), 169.05 (C, C-aromatic) ppm.

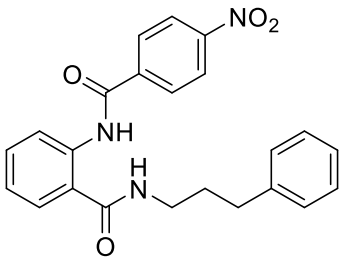
MS (ESI)$^-$: 424.3 $[M-H]^-$

m.p.: 104 – 106 °C

Synthesis of 2-(4-nitrobenzamido)-*N*-(3-phenylpropyl)benzamide (151)

Chemical Formula: $C_{23}H_{21}N_3O_4$

Molecular Weight: 403.44



Procedure: 2

Yield: 43 %

State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 1.93 – 2.11 (m, 2H), 2.80 (t, J = 7.4 Hz, 2H), 3.56 (dd, J = 6.9, 12.7 Hz, 2H), 6.25 (s, 1H), 7.11 – 7.18 (m, 1H), 7.22 – 7.28 (m, 4H), 7.31 – 7.38 (m, 2H), 7.57 (dd, J = 4.3, 11.5 Hz, 1H), 8.16 – 8.26 (m, 2H), 8.34 – 8.45 (m, 2H), 8.79 – 8.88 (m, 1H), 12.58 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.73 (CH_2, C-aliphatic), 33.64 (CH_2, C-aliphatic), 40.01 (CH_2, C-aliphatic), 120.07 (C, C-aromatic), 121.52 (CH, C-aromatic), 123.41 (CH, C-aromatic), 123.99 (CH, C-aromatic), 126.31 (CH, C-aromatic), 126.37 (CH, C-aromatic), 128.39 (CH, C-aromatic), 128.60 (CH, C-aromatic), 128.76 (CH, C-aromatic), 132.98 (CH, C-aromatic), 139.65 (C, C-aromatic), 140.47 (C, C-aromatic), 141.16 (C, C-aromatic), 149.78 (C, C-aromatic), 163.28 (C, C-aromatic), 169.00 (C, C-aromatic) ppm.

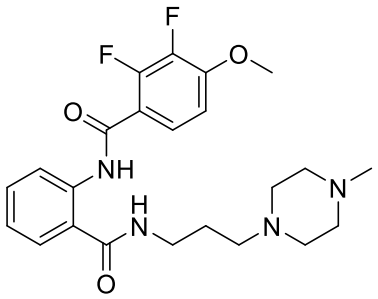
MS (ESI)$^-$: 402.2 $[M-H]^-$

m.p.: 122 – 124 °C

Synthesis of 2,3-difluoro-4-methoxy-N-(2-((3-(4-methylpiperazin-1-yl)propyl)carbamoyl)phenyl)benzamide (152)

Chemical Formula: $C_{23}H_{28}F_2N_4O_3$

Molecular Weight: 446.50



Procedure: 2

Yield: 66 %

State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 1.71 (s, 2H), 1.74 – 1.86 (m, 2H), 2.31 (s, 3H), 2.51 (dd, J = 22.5, 29.0 Hz, 4H), 2.60 – 2.75 (s, 3H), 3.59 (dd, J = 5.7, 10.5 Hz, 2H), 3.98 (d, J = 5.4 Hz, 4H), 6.74 – 6.97 (m, 1H), 7.12 – 7.20 (m, 2H), 7.46 – 7.58 (m, 1H), 7.70 – 7.86 (m, 1H), 8.59 – 8.82 (m, 1H), 8.76 – 8.94 (m, 1H), 12.10 (d, J = 5.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.53 (CH_2, C-aliphatic), 41.29 (CH_2, C-aliphatic), 46.05 (CH_3, C-aliphatic), 53.30 (CH_2, C-aliphatic), 55.03 (CH_2, C-aliphatic), 56.71 (CH_3, C-aliphatic), 58.63 (CH_2, C-aliphatic), 108.05 (CH, C-aromatic), 121.65 (C, C-aromatic), 122.22 (CH, C-aromatic), 123.00 (CH, C-aromatic), 125.28 (CH, C-aromatic), 125.31 (CH, C-aromatic), 125.34 (CH, C-aromatic), 127.35 (C, C-aromatic), 132.21 (C, C-aromatic), 139.32 (C, C-aromatic), 148.84 (C, C-aromatic), 151.79 (C, C-aromatic), 161.44 (C, C-aromatic), 168.64 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 136.59, -158.54 ppm.

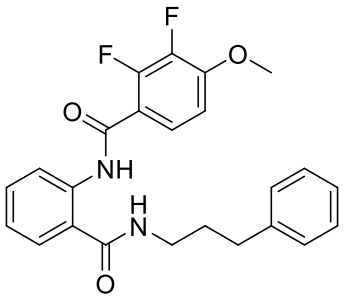
MS (ESI)$^-$: 445.3 $[M-H]^-$

m.p.: 105 – 107 °C

Synthesis of 2, 3-difluoro-4-methoxy-*N*-(2-((3-phenylpropyl)carbamoyl)phenyl)benzamide (153)

Chemical Formula: $C_{24}H_{22}F_2N_2O_3$

Molecular Weight: 424.45



Procedure: 2

Yield: 55 %

State: pale yellow powder

^{1}H-NMR ($CDCl_3$): δ 1.93 – 2.08 (m, 2H), 2.77 (t, J = 7.5 Hz, 2H), 3.53 (dd, J = 6.9, 12.8 Hz, 2H), 3.99 (s, 3H), 6.16 (s, 1H), 6.81 – 6.94 (m, 1H), 7.10 (td, J = 7.7, 1.1 Hz, 1H), 7.28 (tq, J = 8.0, 15.6 Hz, 6H), 7.47 – 7.59 (m, 1H), 7.81 (td, J = 2.3, 9.0 Hz, 1H), 8.70 (d, J = 7.8 Hz, 1H), 11.68 (d, J = 6.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.89 (CH_2, C-aliphatic), 33.56 (CH_2, C-aliphatic), 39.84 (CH_2, C-aliphatic), 56.72 (CH_3, C-aliphatic), 108.09 (CH, C-aromatic), 116.06 (C, C-aromatic), 122.01 (C, C-aromatic), 122.44 (CH, C-aromatic), 123.30 (CH, C-aromatic), 125.44 (CH, C-aromatic), 126.18 (CH, C-aromatic), 126.41 (CH, C-aromatic), 128.40 (CH, C-aromatic), 128.66 (CH, C-aromatic), 132.27 (CH, C-aromatic), 138.90 (C, C-aromatic), 141.32 (C, C-aromatic), 141.82 (C, C-aromatic), 148.79 (C, C-aromatic), 151.81 (C, C-aromatic), 161.35 (C, C-aromatic), 168.63 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 136.57, 158.49 ppm.

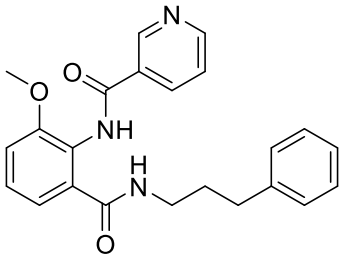
MS (ESI)$^-$: 423.2 $[M-H]^-$

m.p.: (from ethyl acetate/*n*-hexane) 106 – 108 °C

Synthesis of *N*-(2-methoxy-6-((3-phenylpropyl)carbamoyl)phenyl)nicotinamide (154)

Chemical Formula: $C_{23}H_{23}N_3O_3$

Molecular Weight: 389.46



Procedure: 2

Yield: 86 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.80 (dt, J = 7.3, 14.6 Hz, 2H), 2.51 – 2.63 (m, 2H), 3.32 (dd, J = 7.0, 13.0 Hz, 2H), 3.81 (s, 3H), 6.23 (s, 1H), 6.91 (dd, J = 1.2, 7.8 Hz, 3H), 6.99 (dd, J = 1.1, 8.4 Hz, 3H), 7.05 – 7.14 (m, 2H), 7.32 (dd, J = 4.6, 7.6 Hz, 1H), 8.11 – 8.18 (m, 1H), 8.69 (d, J = 3.4 Hz, 1H), 8.99 (s, 1H), 9.12 (d, J = 1.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.96 (CH_2, C-aliphatic), 33.31 (CH_2, C-aliphatic), 39.65 (CH_2, C-aliphatic), 56.19 (CH_3, C-aliphatic), 113.84 (CH, C-aromatic), 119.07 (CH, C-aromatic), 123.46 (CH, C-aromatic), 124.17 (C, C-aromatic), 125.85 (CH, C-aromatic), 126.03 (CH, C-aromatic), 127.34 (CH, C-aromatic), 128.38 (CH, C-aromatic), 128.50 (C, C-aromatic), 129.89 (C, C-aromatic), 132.65 (CH, C-aromatic), 135.42 (C, C-aromatic), 141.29 (CH, C-aromatic), 149.01 (CH, C-aromatic), 152.57 (C, C-aromatic), 154.33 (C, C-aromatic), 164.71 (C, C-aromatic), 168.36 (C, C-aromatic) ppm.

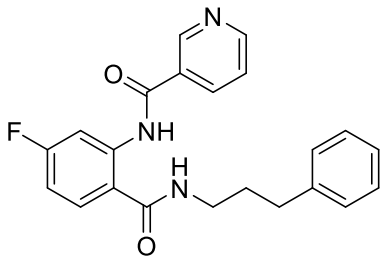
MS (ESI)$^-$: 388.17 $[M-H]^-$

m.p.: (from ethyl acetate/*n*-hexane) 133 – 135 °C

Synthesis of *N*-(5-fluoro-2-((3-phenylpropyl)carbamoyl)phenyl)nicotinamide (155)

Chemical Formula: $C_{22}H_{20}FN3O_2$

Molecular Weight: 377.42



Procedure: 2

Yield: 67 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.94 – 2.12 (m, 2H), 2.80 (t, *J* = 7.3 Hz, 2H), 3.55 (dd, *J* = 6.8, 12.6 Hz, 2H), 6.18 (s, 1H), 6.78 (ddd, *J* = 2.6, 7.5, 8.8 Hz, 1H), 7.05 – 7.37 (m, 4H), 7.-39-7.45 (m, 3H), 8.32 (d, *J* = 7.9 Hz, 1H), 8.65 (dd, *J* = 2.6, 11.6 Hz, 1H), 8.83 (s, 1H), 9.33 (s, 1H), 12.71 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.67 (CH_2, C-aliphatic), 33.73 (CH_2, C-aliphatic), 40.07 (CH_2, C-aliphatic), 108.64 (CH, C-aromatic), 108.86 (CH, C-aromatic), 110.00 (CH, C-aromatic), 110.18 (CH, C-aromatic), 126.30 (C, C-aromatic), 128.16 (CH, C-aromatic), 128.24 (CH, C-aromatic), 128.41 (CH, C-aromatic), 128.78 (CH, C-aromatic), 134.90 (CH, C-aromatic), 104.97 (C, C-aromatic), 141.24 (CH, C-aromatic), 141.97 (C, C-aromatic), 142.07 (CH, C-aromatic), 149.16 (CH, C-aromatic), 152.66 (C, C-aromatic), 163.96 (C, C-aromatic), 165.9 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 103.70 ppm.

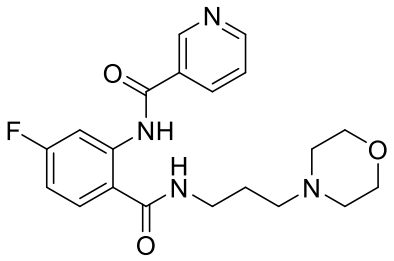
MS (ESI)$^{-}$: 376.2 $[M-H]^-$

m.p.: (from ethyl acetate/*n*-hexane) 96 – 98 °C

Synthesis of N-(5-fluro-2-(3-morpholinopropylcarbamoyl)phenyl)nicotinamide (156)

Molecular Formula: $C_{20}H_{23}FN_4O_3$

Molecular Weight: 386.42



Procedure: 2

Yield: 57%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.72 – 1.88 (m, 2H), 2.36 – 2.47 (m, 2H), 2.53 (bs, 4H), 3.52 (dd, J = 5.8, 10.6 Hz, 2H), 3.65 (s, 4H), 6.79 (ddd, J = 2.6, 7.4, 8.8 Hz, 1H), 7.37 (s, 1H), 7.55 (dd, J = 6.2, 8.5 Hz, 1H), 8.19 – 8.30 (m, 1H), 8.61 (dd, J = 2.6, 11.6 Hz, 1H), 8.64 (d, J = 14.1 Hz, 1H), 8.71 (dd, J = 1.5, 4.8 Hz, 1H), 9.23 (d, J = 1.8 Hz, 1H), 12.89 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 23.15 (CH_2, C-aliphatic), 41.20 (CH_2, C-aliphatic), 53.84 (CH_2, C-aliphatic), 59.11 (CH_2, C-aliphatic), 66.86 (CH_2, C-aliphatic), 108.76 (CH, C-aromatic), 109.97 (CH, C-aromatic), 116.08 (C, C-aromatic), 123.51 (CH, C-aromatic), 128.62 (CH, C-aromatic), 130.18 (C, C-aromatic), 134.91 (CH, C-aromatic), 142.23 (C, C-aromatic), 149.23 (CH, C-aromatic), 152.65 (CH, C-aromatic), 163.95 (C, C-aromatic), 165.97 (C, C-aromatic), 168.42 (C, C-aromatic) ppm.

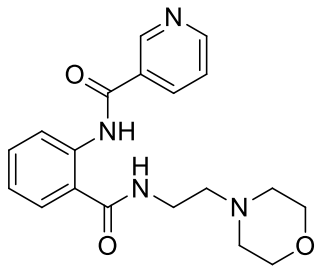
^{19}F-NMR ($CDCl_3$): δ -103.82 ppm.

MS (ESI)$^-$: 385.2 $[M-H]^-$

m.p: (from dichloromethane/*n*-hexane) 127-129 °C

Synthesis of N-(2-(2-morpholinoethylcarbamoyl)phenyl)nicotinamide (157)

Chemical Formula: $C_{19}H_{22}N_4O_3$
Molecular Weight: 354.40



Procedure: 2

Yield: 48%

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.57 (s, 4H), 2.68 (t, J =6 Hz, 2H), 3.60 (q, J =4.5 Hz, 2H), 3.78 (t, J =4.5 Hz, 4H), 7.14 (s, 1H), 7.2 (td, J =1.5, 8 Hz, 1H), 7.47 (td, J =0.5, 4.5 Hz, 1H), 7.56-7.61 (m, 2H), 8.33 (dt, J =8.2 Hz, 1H), 8.80 (dd, J =1.1, 5.5 Hz, 1H), 8.84 (d, J =9.1 Hz, 1H), 9.32 (d, J =1.5 Hz, 1H), 12.53 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.92 (CH_2, C-aliphatic), 53.29 (CH_2, C-aliphatic), 56.60 (CH_2, C-aliphatic), 66.84 (CH_2, C-aliphatic), 120.03 (C, C-aromatic), 121.64 (CH, C-aromatic), 123.30 (CH, C-aromatic), 123.46 (CH, C-aromatic), 126.56 (CH, C-aromatic), 130.52 (C, C-aromatic), 132.94 (CH, C-aromatic), 134.91 (CH, C-aromatic), 139.94 (C, C-aromatic), 149.13 (CH, C-aromatic), 152.46 (CH, C-aromatic), 163.73 (C, C-aromatic), 169.07 (C, C-aromatic) ppm.

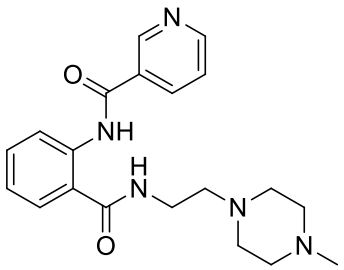
MS (ESI)$^+$: 355.2 $[M+H]^+$

m.p.: (from ethanol/water) 84-86 ^{o}C

Synthesis of N-(2-(2-(4-methylpiperazin-1-yl)ethylcarbamoyl)phenyl)nicotinamide (**158**)

Chemical Formula: $C_{20}H_{25}N_5O_2$

Molecular Weight: 367.20



Procedure: 2

Yield: 27 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.32 (s, 3H), 2.24-2.63 (m, 8 H), 2.65 (t, J =5.7 Hz, 2H), 3.55 (q, J =5.5 Hz, 2H), 7.14 (s, 1H), 7.17 (td, J =0.95, 7.45 Hz, 1H), 7.45 (dd, J =5, 7.8 Hz, 1H), 7.52-7.59 (m, 2H), 8.32 (dt, J =1.95, 8.05 Hz, 1H), 8.78 (dd, J =1.55, 4.9 Hz, 1H), 8.83 (d, J =8.1 Hz, 1H), 9.31 (d, J = 1.95 Hz, 1H), 12.56 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 29.68 (CH_2, C-aliphatic), 45.99 (CH_3, C-aliphatic), 52.74 (CH_2, C-aliphatic), 55.17 (CH_2, C-aliphatic), 55.83 (CH_2, C-aliphatic), 120.15 (C, C-aromatic), 121.58 (CH, C-aromatic), 123.25 (CH, C-aromatic), 123.44 (CH, C-aromatic), 126.56 (CH, C-aromatic), 130.50 (C, C-aromatic), 132.81 (CH, C-aromatic), 134.83 (CH, C-aromatic), 139.92 (C, C-aromatic), 149.20 (CH, C-aromatic), 152.43 (CH, C-aromatic), 163.73 (C, C-aromatic), 168.98 (C, C-aromatic) ppm.

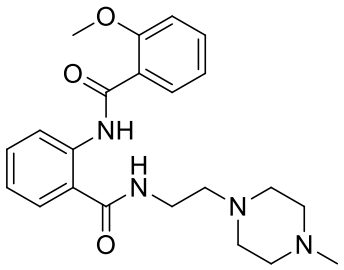
MS (ESI)$^+$: 368.2 $[M+H]^+$

m.p.: (from ethanol/water) 98-100 ^oC

Acc. Mass calcul. : 368, 2081; **found:** 368.2083.

Synthesis of 2-methoxy-N-(2-((2-(4-methylpiperazin-1-yl)ethyl)carbamoyl)phenyl)benzamide (159)

Chemical Formula: $C_{22}H_{28}N_4O_3$
Molecular Weight: 396.22



Procedure: 2

Yield: 78%
State: yellow solid

^{1}H-NMR ($CDCl_3$): δ 2.31 (s, 3H), 2.36-2.59 (m, 8 H), 2.61 (t, J =6 Hz, 2H), 3.55 (q, J =5.5 Hz, 2H), 6.73 (s, 1H), 7.03 (d, J =8 Hz, 1H), 7.10 (t, J =1, 8 Hz, 1H), 7.14 (t, J =8 Hz, 1H), 7.44-7.54 (m, 3H), 8.22 (dd, J =1.5, 8 Hz, 1H), 8.71 (d, 1H), 11.76 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.25 (CH_2, C-aliphatic), 45.88 (CH_3, C-aliphatic), 52.69 (CH_2, C-aliphatic), 55.04 (CH_2, C-aliphatic), 55.69 (CH_3, C-aliphatic), 56.23 (CH_2, C-aliphatic), 11.38 (CH, C-aromatic), 120.92 (CH, C-aromatic), 122.51 (C, C-aromatic), 123.11 (CH, C-aromatic), 123.18 (CH, C-aromatic), 124.45 (C, C-aromatic), 126.64 (CH, C-aromatic), 131.63 (CH, C-aromatic), 132.28 (CH, C-aromatic), 133.07 (CH, C-aromatic), 138.41 (C, C-aromatic), 157.75 (C, C-aromatic), 164.21 (C, C-aromatic), 168.71 (C, C-aromatic) ppm.

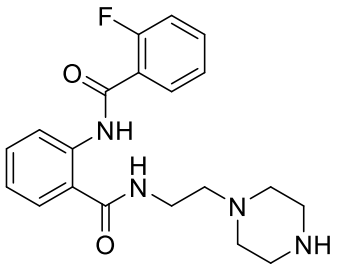
m.p.: (from ethanol/water) 110-112 ^oC

MS (ESI)$^+$: 397.2 $[M+H]^+$

Synthesis 2-fluoro-*N*-(2-((2-(piperazin-1-yl)ethyl)carbamoyl)phenyl)benzamide (160)

Chemical Formula: $C_{20}H_{23}FN_4O_2$

Molecular Weight: 370,43



Procedure: 2

Yield: 45 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 1.70 (s, 1H), 2.26 (s, 2H), 2.31-2.58 (m, 4H), 2590 (t, *J* =6.1 Hz, 2H), 3.56 (q, *J* = 4.5 Hz, 4H), 7.11 (s, 1H), 7.15 (td, *J* =1.05, 7.65 Hz, 1H), 7.20 (dd, *J* =0.9, 8.3 Hz, 1H), 7.26 (td, *J* =1, 7.55 Hz, 1H), 7.40-7.53 (m, 3H), 8.02 (d, *J* = 8 Hz, 1H), 8.71 (d, *J* =8.0 Hz, 1H), 11.80 (d, *J* =6.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.38 (CH_2, C-aliphatic), 52.72 (CH_2, C-aliphatic), 55.10 (CH_2, C-aliphatic), 56.12 (CH_2, C-aliphatic), 116.50 (CH, C-aromatic), 121.98 (C, C-aromatic), 122.31 (CH, C-aromatic), 122.86 (CH, C-aromatic), 123.31 (CH, C-aromatic), 124.57 (CH, C-aromatic), 126.62 (CH, C-aromatic), 131.41 (CH, C-aromatic), 132.29 (CH, C-aromatic), 139.12 (C, C-aromatic), 159.36 (C, C-aromatic), 161.39 (C, C-aromatic), 162.41 (C-C-aromatic), 168.59 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.19 ppm.

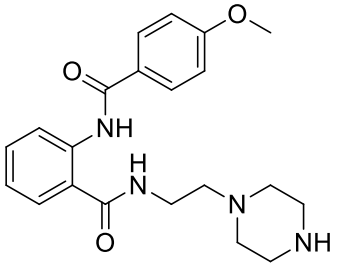
MS(ESI)$^{+}$: 371.2 $[M+H]^{+}$

m.p.: (from ethanol/water) 104-106 ^{o}C

Synthesis of 2-(4-methoxybenzamido)-N-(2-(piperazin-1-yl)ethyl)benzamide (161)

Chemical formula: $C_{21}H_{26}N_4O_3$

Molecular Weight: 382.20



Procedure: 2

Yield: 23 %

State: yellow solid

^{1}H-NMR ($CDCl_3$): δ 2.60-2.48 (m, 5H), 2.63 (t, J =5.7 Hz, 2H), 2.91-2.99 (m, 4H), 3.56 (q, J =4.55 Hz, 2H), 3.89 (s, 3H), 7.01 (d, J =6.9 Hz, 2H), 7.07-7.16 (m, 2H), 7.49-7.58 (m, 2H), 8.03 (d, J =8.05 Hz, 2H), 8.83 (d, J =8.05 Hz, 1H), 12.19 (s, 1H) ppm.

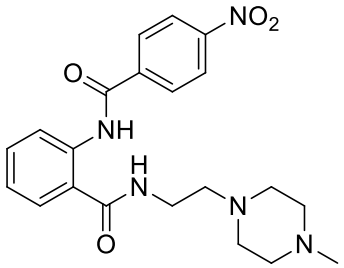
^{13}C-NMR ($CDCl_3$): δ 36.10 (CH_2, C-aliphatic), 45.93 (CH_2, C-aliphatic), 53.67 (CH_2, C-aliphatic), 55.45 (CH_3, C-aliphatic), 56.46 (CH_2, C-aliphatic), 113.96 (CH, C-aromatic), 120.25 (C, C-aromatic), 121.53 (CH, C-aromatic), 122.60 (CH, C-aromatic), 126.57 (CH, C-aromatic), 127.23 (C, C-aromatic), 129.31 (CH, C-aromatic), 132.68 (CH, C-aromatic), 140.33 (C, C-aromatic), 162.47 (C, C-aromatic), 165.18 (C, C-aromatic), 169.20 (C, C-aromatic) ppm.

MS (ESI)$^+$: 383.2 $[M+H]^+$

m.p.: (from ethanol/water) 90-92 °C

Synthesis of N-(2-(4-methylpiperazin-1-yl)ethyl)-2-(4-nitrobenzamido)benzamide (162)
Chemical Formula: $C_{21}H_{25}N_5O_4$

Molecular Weight: 411.19



Procedure: 2

Yield: 85%

State: white solid

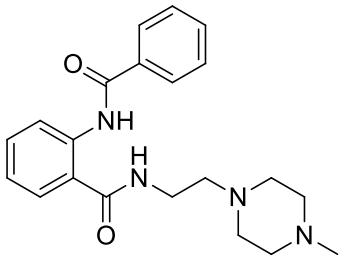
^{1}H-NMR ($CDCl_3$): δ 2.34 (s, 3H), 2.42-2.65 (m, 8 H), 2.67 (t, J =5.9 Hz, 2H), 3.57 (q, J =5.35 Hz, 2H), 7.18 (s, 1H), 7.21 (td, J =1.15, 7.35 Hz, 1H), 7.55-7.61 (m, 2H), 8.23 (d, J =8.9 Hz, 2H), 8.37 (d, J =8.95 Hz, 2H), 8.85 (dd, J =0.9, 8.45 Hz, 1H), 12.73 (s,1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.27 (CH_2, C-aliphatic), 45.94 (CH_3, C-aliphatic), 52.68 (CH_2, C-aliphatic), 55.11 (CH_2, C-aliphatic), 55.79 (CH_2, C-aliphatic), 119.99 (C, C-aromatic), 121.51 (CH, C-aromatic), 123.51 (CH, C-aromatic), 123.96 (CH, C-aromatic), 126.61 (CH, C-aromatic), 128.59 (CH, C-aromatic), 132.97 (CH, C-aromatic), 139.87 (C, C -aromatic), 140.51 (C, C-aromatic), 149.77 (C, C-aromatic), 163.27 (C, C-aromatic), 168.98 (C, C-aromatic), ppm.

MS (ESI): 412.2 $[M+H]^+$

m.p. (from ethanol/water): 121-123 ^oC

Synthesis of 2-benzamido-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (163)
Chemical Formula: $C_{21}H_{26}N_4O_2$
Molecular Weight: 366.21



Procedure: 2

Yield: 88 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.34 (s, 3H), 2.38-2.64 (m, 8H), 2.66 (t, J =5.45 Hz, 2H), 3.57 (q, J =5.45 Hz, 2H), 7.07 (s, 1H), 7.16 (td, J =0.9, 7.6 Hz, 1H), 7.51-7.58 (m, 5H), 8.07 (dd, J =1.3, 8.2 Hz, 2H), 8.86 (dd, J =0.8, 8.5 Hz, 1H), 12.28 (s, 1H) ppm.

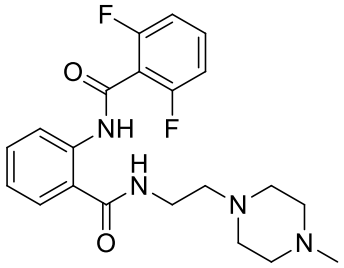
^{13}C-NMR ($CDCl_3$): δ 36.27 (CH_2, C-aliphatic), 45.92 (CH_3, C-aliphatic), 52.66 (CH_2, C-aliphatic), 55.10 (CH_2, C-aliphatic), 55.90 (CH_2, C-aliphatic), 120.42 (C, C-aromatic), 121.62 (CH, C-aromatic), 122.84 (CH, C-aromatic), 126.56 (CH, C-aromatic), 127.41 (CH, C-aromatic), 128.75 (CH, C-aromatic), 131.78 (CH, C-aromatic), 132.69 (CH, C-aromatic), 134.92 (C, C-aromatic), 140.17 (C, C-aromatic), 165.57 (C, C-aromatic), 169.11 (C, C-aromatic) ppm.

MS (ESI)$^+$: 367.2 $[M+H]^+$

m.p.: (from ethanol/water) 79-81^{o}C

Synthesis of 2, 6-difluoro-N-(2-(2-(4-methylpiperazin-1-yl)ethylcarbamoyl) phenyl) benzamide (164)

Chemical Formula: $C_{21}H_{24}F_2N_4O_2$
Molecular Weight: 402.19



Procedure: 2

Yield: 54%

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.32 (s, 3H), 2.38-2.60 (m, 8H), 2.62 (t, J =6.5, 2H), 3.50 (q, J =6, 2H), 6.99 (t, J =8, 2H), 7.08 (s, 1H), 7.18 (t, J =7.5, 1H), 7.40 (q, J =6.5, 1H), 7.52-7.57 (m, 2H), 8.81 (d, J =8.05, 1H), 11.8 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.23 (CH_2, C-aliphatic), 56.43 (CH_3, C-aliphatic), 50.59 (CH_2, C-aliphatic), 52.66 (CH_2, C-aliphatic), 55.08 (CH_2, C-aliphatic), 55.92 (CH_2, C-aliphatic), 114.13 (C, C-aromatic), 122.76 (C, C-aromatic), 129.91 (CH, C-aromatic), 143.33 (C, C-aromatic), 149.33 (C, C-aromatic), 156.44 (C, C-aromatic), 158.95 (C, C-aromatic), 161.69 (C, C-aromatic), 168.64 (C, C-aromatic) ppm.

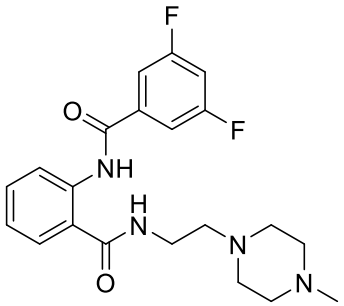
^{19}F-NMR ($CDCl_3$): - 107.75 ppm.

MS (ESI): 403.1 $[M+H]^+$

m.p.: (from ethanol/water) 80-82 ^oC

Synthesis of 3, 5-difluoro-N-(2-(2-(4-methylpiperazin-1-yl) ethylcarbamoyl) phenyl)benzamide (165)

Chemical Formula: $C_{21}H_{24}F_2N_4O_2$
Molecular Weight: 402.19



Procedure: 2

Yield: 65 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.33 (s, 3H), 2.37-2.64 (m, 8 H), 2.66 (t, J =5.85 Hz, 2H), 3.57 (q, J =5.4 Hz, 2H), 7.00 (tt, J =2.1, 8.65 Hz, 1H), 7.11 (s, 1H), 7.19 (td, J =0.9, 7.2 Hz, 1H), 7.55 (d, J =7.80 Hz, 1H), 7.59 (d, J =7.85 Hz, 3H), 8.8 (d, J =8.1 Hz, 1H), 12.51 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.25 (CH_2, C-aliphatic), 45.96 (CH_3, C-aliphatic), 55.70 (CH_2, C-aliphatic), 55.13 (CH_2, C-aliphatic), 55.85 (CH_2, C-aliphatic), 107.12 (CH, C-aromatic), 110.59 (CH, C-aromatic), 110.75 (CH, C-aromatic), 120.13 (C, C-aromatic), 121.57 (CH, C-aromatic), 123.33 (CH, C-aromatic), 126.54 (CH, C-aromatic), 132.85 (CH, C-aromatic), 139.84 (C, C-aromatic), 159.18 (C, C-aromatic), 162.54 (C, C-aromatic), 163.11 (C, C-aromatic), 164.08 (C, C-aromatic), 168.92 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): - 107.78 ppm.

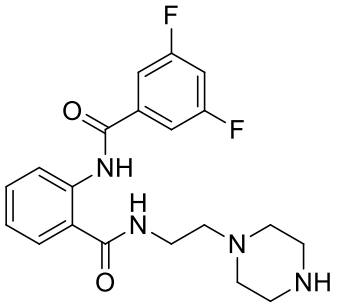
MS(ESI)$^+$: 403.2 $[M+H]^+$

m.p.: (from ethanol/water) 110-112 ^oC

Synthesis of 3, 5-difluoro-N-(2-(2-(piperazin-1-yl)ethylcarbamoyl)phenyl) benzamide (166)

Chemical formula: $C_{20}H_{22}F_2N_4O_2$

Molecular Weight: 388.1



Procedure: 2

Yield: 37%

State: yellow solid

^{1}H-NMR ($CDCl_3$): δ 2.44-2-59 (m, 5H), 2.64 (t, J = 5.3 Hz, 2H), 2.95 (t, J = 4.55 Hz, 4H), 3.57 (q, J = 4.15 Hz, 2H), 7.00 (t, J = 9.8 Hz, 1H), 7.18 (q, J = 7.3 Hz, 2H), 7.52 - 7.61 (m, 4H), 8.78 (d, J = 7.9 Hz, 1H), 12.5 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.06 (CH_2, C-aliphatic), 45.82 (CH_2, C-aliphatic), 53.57 (CH_2, C-aliphatic), 56.47 (CH_2, C-aliphatic), 107.16 (CH, C-aromatic), 110.59 (CH, C-aromatic), 110.75 (CH, C-aromatic), 120.09 (C, C-aromatic), 121.57 (CH, C-aromatic), 123.39 (CH, C-aromatic), 126.59 (CH, C-aromatic), 132.90 (CH, C-aromatic), 138.47 (C, C-aromatic), 139.79 (C, C-aromatic), 163.03 (C, C-aromatic), 164.11 (C, C-aromatic), 168.96 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): - 107.85 ppm.

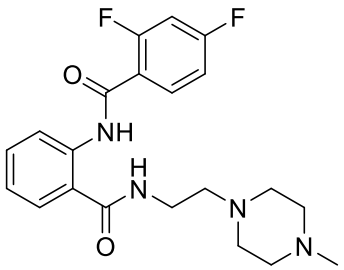
MS (ESI)$^+$: 389.2 $[M+H]^+$

m.p.: (from ethanol/water) 67-69 ˚C

Synthesis of 2, 4-difluoro-N-(2-(2-(4-methylpiperazin-1-yl)ethylcarbamoyl) phenyl)benzamide (167)

Chemical Formula: $C_{21}H_{24}F_2N_4O_2$

Molecular Weight: 402.19



Procedure: 2

Yield: 85 %

State: yellow solid

^{1}H-NMR ($CDCl_3$): δ 2.32 (s, 3H), 2.36-2.61 (m, 8H), 2.64 (t, J =6.1 Hz, 2H), 3.54 (q, J =5.45 Hz, 2H), 6.92-6.97 (m, 2H), 7.02 (t, J =8.2 Hz, 1H), 7.2 (td, J =1, 7.6 Hz, 1H), 7.51 (dd, J =1.25, 7.8 Hz, 1H), 7.55 (t, J =8.05 Hz, 1H), 8.11 (q, J =8.85 Hz, 1H), 8.75 (d, J =8.3 Hz, 1H), 11.89 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.25 (CH_2, C-aliphatic), 45.98 (CH_3, C-aliphatic), 52.73 (CH_2, C-aliphatic), 55.16 (CH_2, C-aliphatic), 55.92 (CH_2, C-aliphatic), 104.67 (CH, C-aromatic), 112.17 (CH, C-aromatic), 121.83 (C, C-aromatic), 122.46 (CH, C-aromatic), 123.44 (CH, C-aromatic), 126.59 (CH, C-aromatic), 132.35 (CH, C-aromatic), 133.31 (CH, C-aromatic), 139.10 (C, C-aromatic), 161.32 (C, C-aromatic), 161.93 (C, C-aromatic), 163.85 (C, C-aromatic), 165.88 (C, C-aromatic), 168.59 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -104.00, -107.63 ppm.

m.p.: (from ethanol/water): 121-122 ^oC

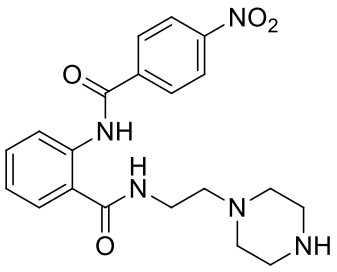
MS (ESI)$^+$: 403.2 $[M+H]^+$

Accurate Mass calculated : 385.2034; **found:** 385.2036.

Synthesis of 2-(4-nitrobenzamido)-N-(2-(piperazin-1-yl)ethyl)benzamide (168)

Chemical formula: $C_{20}H_{23}N_5O_4$

Molecular Weight: 397.1



Procedure: 2

Yield: 13 %

State: yellow solid

^{1}H-NMR ($CDCl_3$): δ 2.6 (s, 5H), 2.67 (t, J = 6.05 Hz, 2H), 3.00 (t, J = 4.7 Hz, 4H), 3.58 (q, J = 5.25 Hz, 2H), 7.2 (s, 1H), 7.22 (t, J = 6.75 Hz, 1H), 7.6 (q, J = 7.5 Hz, 2H), 8.24 (d, J = 9 Hz, 2H), 8.39 (d, J = 9 Hz, 2H), 8.85 (d, J = 8.25 Hz, 1H), 12.73 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.07 (CH_2, C-aliphatic), 45.71 (CH_2, C-aliphatic), 53.35 (CH_2, C-aliphatic), 56.33 (CH_2, C-aliphatic), 119.80 (C, C-aromatic), 121.54 (CH, C-aromatic), 123.57 (CH, C-aromatic), 123.99 (CH, C-aromatic), 126.61 (CH, C-aromatic), 128.61 (CH, C-aromatic), 133.06 (CH, C-aromatic), 139.85 (C, C-aromatic), 140.50 (C, C-aromatic), 149.76 (C, C-aromatic), 163.29 (C, C-aromatic), 169.02 (C, C-aromatic) ppm.

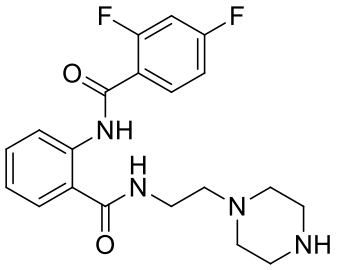
MS (ESI)$^+$: 398.2 $[M+H]^+$

m.p.: (from ethanol/water) 97-99 °C

Synthesis of 2, 4-difluoro-N-(2-(2-(piperazin-1-yl)ethylcarbamoyl) phenyl) benzamide (169)

Chemical formula: $C_{20}H_{22}F_2N_4O_2$

Molecular Weight: 388.17



Procedure: 2

Yield: 12 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 1.25 (s, 1H), 2.49 (s, 4H), 2.58 (t, J = 5.85 Hz, 2H), 2.9 (t, J = 4.75 Hz, 4H), 3.51 (q, J = 5.35 Hz, 2H), 6.92 (t, J =9.8, 1H), 6.99 (t, J =7.95 Hz, 1H), 7.09-7.16 (m, 2H), 7.47-7.54 (m, 2H), 8.07 (q, J = 6.9 Hz, 1H), 8.7 (d, J = 8.5 Hz, 1H), 11.86 (d, J = 6.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.11 (CH_2, C-aliphatic), 44.86 (CH_2, C-aliphatic), 52.01 (CH_2, C-aliphatic), 56.42 (CH_2, C-aliphatic), 104.70 (CH, C-aromatic H), 106.20 (CH, C-aromatic), 112.26 (CH, C-aromatic), 121.77 (C, C-aromatic), 123.54 (CH, C-aromatic), 126.68 (CH, C-aromatic), 132.45 (CH, C-aromatic), 133.35 (CH, C-aromatic), 138.99 (C, C-aromatic), 159.81 (C, C-aromatic), 161.29 (C, C-aromatic), 163.87 (C, C-aromatic), 165.95 (C, C-aromatic), 168.68 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -103.75, -107.45 ppm.

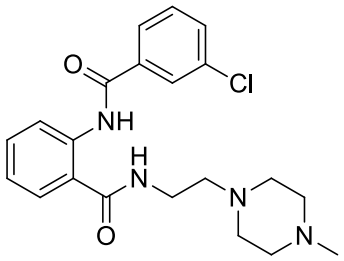
MS (ESI)$^+$: 389.2 $[M+H]^+$

m.p.: (from ethanol/water) 83-85 ˚C

Synthesis of 2-(3-chlorobenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl) benzamide (170)

Chemical formula: $C_{21}H_{25}ClN_4O_2$

Molecular Weight: 400.17



Procedure: 2

Yield: 47 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.35 (s, 3H), 2.40-2.62 (m, 8H), 2.68 (t, J =5.6 Hz, 2H), 7.13 (s, 1H), 3.57 (q, J =5 Hz, 2H), 7.18 (t, J =7.55 Hz, 1H), 7.49 (d, J =8.1 Hz, 2H), 7.53-7.59 (m, 2H), 8.01 (d, J =8.1 Hz, 2H), 8.83 (d, J =8.1 Hz, 1H), 12.39 (s,1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.21 (CH_2, C-aliphatic), 45.85 (CH_3, C-aliphatic), 52.54 (CH_2, C-aliphatic), 55.00 (CH_2, C-aliphatic), 55.84 (CH_2, C-aliphatic), 120.13 (C, C-aromatic), 121.53 (CH, C-aromatic), 123.04 (CH, C-aromatic), 126.61 (CH, C-aromatic), 128.58 (CH, C-aromatic), 128.87 (CH, C-aromatic), 129.02 (CH, C-aromatic), 132.83 (CH, C-aromatic), 133.33 (C, C-aromatic), 138.09 (C, C-aromatic), 140.08 (C, C-aromatic), 164.46 (C, C-aromatic), 169.10 (C, C-aromatic) ppm.

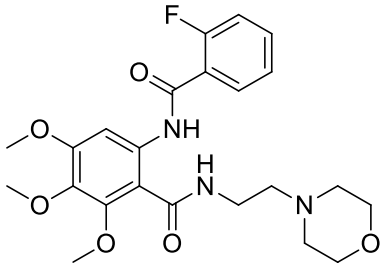
MS(ESI)$^{+}$: 401.2 $[M+1]^{+}$

m.p. : (from ethanol/water) 103-105 ˚C

Synthesis of 6-(2-fluorobenzamido)-2, 3, 4-trimethoxy-N-(2-morpholinoethyl) benzamide (171)

Chemical formula: $C_{23}H_{28}FN_3O_6$

Molecular Weight: 461.20



Procedure: 2

Yield: 41 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.37 (s, 4H), 2.49 (t, J =5.9 Hz, 2H), 3.5 (q, J =5.55 Hz, 2H), 3.59 (t, J =4 Hz, 4H), 3.92 (s, 3H), 3.93 (s, 3H), 3.94 (s, 3H), 6.94 (s, 1H), 7.00 (d, J =0.85 Hz, 1H), 7.23 (dd, J =8.15, 11.9 Hz , 1H), 7.32 (t, J =7.45 Hz, 1H), 7.56 (q, J =7.05 Hz, 1H), 8.16 (t, J =7.9 Hz, 1H), 8.68 (d, J =13.2 Hz,1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.03 (CH_2, C-aliphatic), 53.25 (CH_2, C-aliphatic), 56.29 (CH_3, C-aliphatic), 56.96 (CH_2, C-aliphatic), 61.02 (CH_3, C-aliphatic), 61.16 (CH_3, C-aliphatic), 66.69 (CH_2, C-aliphatic), 106.83 (CH, C-aromatic), 116.38 (CH, C-aromatic), 120.66 (C, C-aromatic), 121.17 (C, C-aromatic), 124.95 (CH, C-aromatic), 128.56 (C, C-aromatic), 132.27 (CH, C-aromatic), 134.03 (CH, C-aromatic), 144.18 (C, C-aromatic), 148.66 (C, C-aromatic), 152.25 (C, C-aromatic), 159.88 (C, C-aromatic), 161.55 (C, C-aromatic), 167.47 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 112.23 ppm.

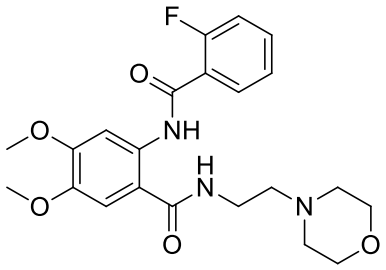
MS (ESI)$^{+}$: 462.20 $[M+H]^{+}$

m.p.: (from ethanol/water) 140-142 ˚C

Synthesis of 2-(2-fluorobenzamido)-4, 5-dimethoxy-N-(2-morpholinoethyl) benzamide (172)

Chemical formula: $C_{22}H_{26}FN_3O_5$

Molecular Weight: 431.19



Procedure: 2

Yield: 26 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.56 (s, 4H), 2.66 (t, J =5.8 Hz, 2H), 3.56 (q, J =5.8 Hz, 2H), 3.75 (t, J =4.25 Hz, 4H), 3.95 (s, 3H), 4.02 (s, 3H), 6.93 (s, 1H), 7.00 (s, 1H), 7.21 (dd, J =0.9, 11.35 Hz, 1H), 7.29 (td, J =1.15, 7.4 Hz, 1H), 7.48-7.53 (m, 1H), 8.04 (dt, J =1.75, 7.65 Hz, 1H), 8.58 (s, 1H), 12.08 (d, J =5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.72 (CH_2, C-aliphatic), 53.23 (CH_2, C-aliphatic), 56.16 (CH_3, C-aliphatic), 56.45 (CH_3, C-aliphatic), 56.63 (CH_2, C-aliphatic), 66.94 (CH_2, C-aliphatic), 105.68 (CH, C-aromatic), 109.52 (CH, C-aromatic), 112.87 (C, C-aromatic), 116.53 (CH, C-aromatic), 124.63 (CH, C-aromatic), 131.30 (CH, C-aromatic), 133.31 (CH, C-aromatic), 134.91 (C, C-aromatic), 144.50 (C, C-aromatic), 152.11 (C, C-aromatic), 159.37 (C, C-aromatic), 161.36 (C, C-aromatic), 162.32 (C, C-aromatic), 168.39 (C, C-aromatic),

^{19}F-NMR ($CDCl_3$): δ -112.60 ppm.

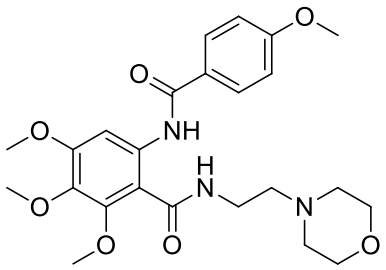
MS (ESI)$^{+}$: 432.2 $[M+H]^{+}$

m.p.: (from ethanol/water) 135-137 °C

Synthesis of 2, 3, 4-trimethoxy-6-(4-methoxybenzamido)-N-(2-morpholinoethyl) benzamide (173)

Chemical formula: $C_{24}H_{31}N_3O_7$

Molecular Weight: 473.22



Procedure: 2

Yield: 38 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.38 (s, 4H), 2.47 (t, J =6.4 Hz, 2H), 3.45 (q, J =5.45 Hz, 2H), 3.63 (t, J =4.2 Hz), 3.89 (s, 3H), 3.90 (s, 3H), 3.92 (s, 3H), 3.94 (s, 3H), 6.93 (s, 1H), 6.99 (d, J =7.3 Hz, 2H), 7.04 (s, 1H), 7.95 (d, J =8.2 Hz, 2H), 8.49 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.04 (CH_2, C-aliphatic), 53.23 (CH_2, C-aliphatic), 55.51 (CH_3, C-aliphatic), 56.30 (CH_3, C-aliphatic), 56.92 (CH_2, C-aliphatic), 61.00 (CH_3, C-aliphatic), 61.11 (CH_3, C-aliphatic), 66.73 (CH_2, C-aliphatic), 106.55 (CH, C-aromatic), 113.93 (CH, C-aromatic), 122.44 (C, C-aromatic), 126.13 (C, C-aromatic), 127.94 (C, C-aromatic), 129.47 (CH, C-aromatic), 144.44 (C, C-aromatic), 149.10 (C, C-aromatic), 151.94 (C, C-aromatic), 162.75 (C, C-aromatic), 166.76 (C, C-aromatic), 167.86 (C, C-aromatic) ppm.

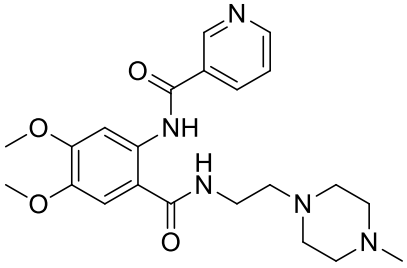
MS (ESI)$^+$: 474.2 $[M+H]^+$

m.p.: (from ethanol/water) 142-144 °C

Synthesis of N-(4,5-dimethoxy-2-(2-(4-methylpiperazin-1-yl)ethylcarbamoyl) phenyl)nicotinamide (174)

Chemical formula: $C_{22}H_{29}N_5O_4$

Molecular Weight: 427.22



Procedure: 2

Yield: 46.84 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ2.28 (s, 3H), 2.30-2.62 (m, 8H), 2.65 (t, J =6.1 Hz, 2H), 3.53 (q, J =5.3 Hz, 2H), 3.91 (s, 3H), 3.99 (s, 3H), 7.03 (s, 1H), 7.27 (s, 1H), 7.44 (dd, J = 4.85, 7.9 Hz, 1H), 8.30 (dt, J = 1.8, 8.15 Hz, 1H), 8.59 (s, 1H), 8.75 (dd, J =1.6, 4.9 Hz, 1H), 9.28 (d, J =2.05 Hz, 1H), 12.86 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.03 (CH_2, C-aliphatic), 46.04 (CH_3, C-aliphatic), 55.62 (CH_2, C-aliphatic), 55.25 (CH_2, C-aliphatic), 55.87 (CH_2, C-aliphatic), 56.15 (CH_3, C-aliphatic), 56.31 (CH_3, C-aliphatic), 104.56 (CH, C-aromatic), 109.27 (CH, C-aromatic), 111.18 (C, C-aromatic), 123.49 (CH, C-aromatic), 130.53 (C, C-aromatic), 134.73 (CH, C-aromatic), 135.75 (C, C-aromatic), 144.38 (C, C-aromatic), 149.10 (CH, C-aromatic), 152.32 (CH, C-aromatic), 152.39 (C, C-aromatic), 163.59 (C, C-aromatic), 168.73 (C, C-aromatic) ppm.

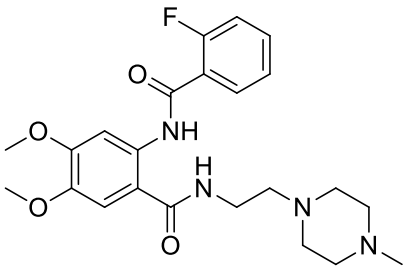
MS (ESI)$^+$: 428.2 $[M+1]^+$

m.p.: (from ethanol/water) 125-127 °C

Synthesis of 2-(2-fluorobenzamido)-4,5-dimethoxy-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (175)

Chemical formula: $C_{23}H_{29}FN_4O_4$

Molecular Weight: 444.22



Procedure: 2

Yield: 89 %

State: white solid

H**^{1}H-NMR (CDCl$_3$):** δ 2.21 (s, 3H), 2.52-2.24 (m, 8H), 2.54 (t, J =5.51 Hz, 2H), 3.44 (q, J =5.45 Hz, 2H), 3.84 (s, 3H), 3.93 (s, 3H), 6.99 (s, 1H), 7.09-7.18 (m, 2H), 7.20 (t, J =6.95 Hz, 1H), 7.43 (q, J =6.35 Hz, 1H), 7.95 (t, J =6.65 Hz, 1H), 8.49 (s, 1H), 12.12 (d, J =5.5 Hz, 1H) ppm.

^{13}C-NMR (CDCl$_3$): δ 36.09 (CH_2, C-aliphatic), 45.95 (CH_3, C-aliphatic), 52.57 (CH_2, C-aliphatic), 55.14 (CH_2, C-aliphatic), 55.99 (CH_2, C-aliphatic), 56.04 (CH_3, C-aliphatic), 56.21 (CH_3, C-aliphatic), 105.43 (CH, C-aromatic), 109.48 (CH, C-aromatic), 112.82 (C, C-aromatic), 116.52 (CH, C-aromatic), 124.57 (CH, C-aromatic), 131.12 (CH, C-aromatic), 133.24 (CH, C-aromatic), 134.75 (C, C-aromatic), 144.37 (C, C-aromatic), 151.81 (C, C-aromatic), 159.25 (C, C-aromatic), 161.25 (C, C-aromatic), 162.25 (C, C-aromatic), 168.32 (C, C-aromatic) ppm.

^{19}F-NMR (CDCl$_3$): δ - 112.95 ppm.

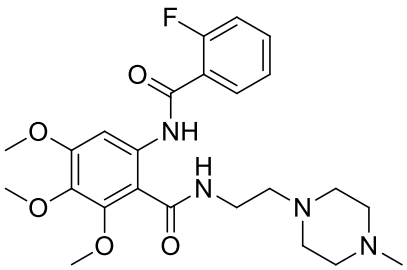
MS (ESI)$^+$: 445.2 $[M+1]^+$

m.p.: (from ethanol/water) 59-61 ˚C

Synthesis of 6-(2-fluorobenzamido)-2, 3, 4-trimethoxy-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (176)

Chemical formula: $C_{24}H_{31}FN_4O_5$

Molecular Weight: 474.23



Procedure: 2

Yield: 51 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.29 (s, 3H), 2.33-2.46 (m, 8H), 2.48-2.58 (m, 2H), 3.49 (q, J =5.1 Hz, 2H), 3.93 (s, 3H), 3.92 (s, 3H), 3.94 (s, 3H), 6.92 (s, 1H), 6.99 (s, 1H), 7.23 (dd, J =8.55, 11.2 Hz, 1H), 7.32 (t, J =6.85 Hz, 1H), 7.57 (q, J =5.7 Hz, 1H), 8.16 (t, J =6.85 Hz, 1H), 8.71 (d, J =13.65 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.40 (CH_2, C-aliphatic), 45.90 (CH_3, C-aliphatic), 52.64 (CH_2, C-aliphatic), 54.91 (CH_2, C-aliphatic), 56.27 (CH_3, C-aliphatic), 56.30 (CH_2, C-aliphatic), 61.01 (CH_3, C-aliphatic), 61.11 (CH_3, C-aliphatic), 106.74 (CH, C-aromatic), 116.36 (CH, C-aromatic), 121.35 (C, C-aromatic), 124.90 (CH, C-aromatic), 128.47 (C, C-aromatic), 132.28 (CH, C-aromatic), 133..33 (CH, C-aromatic), 144.19 (C, C-aromatic), 148.75 (C, C-aromatic), 152.16 (C, C-aromatic), 159.89 (C, C-aromatic), 161.87 (C, C-aromatic), 163.09 (C, C-aromatic), 167.41 (C, C-aromatic) ppm.

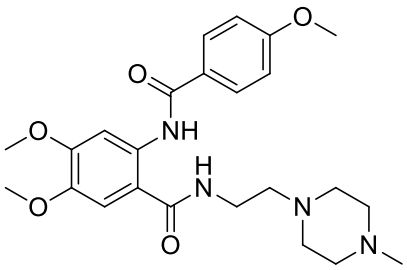
^{19}F-NMR ($CDCl_3$): δ - 112.20 ppm.

MS (ESI)$^{+}$: 475.3 $[M+H]^{+}$

m.p.: (from ethanol/water) 129-131 °C

Synthesis of 4,5-dimethoxy-2-(4-methoxybenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (177)

Chemical formula: $C_{24}H_{32}N_4O_2$
Molecular Weight: 456.24



Procedure: 2

Yield: 6 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ (s, 3H), 2.66-2.35 (m, 8H), 2.68 (t, J =6.15 Hz, 2H), 3.55 (q, J =5.25 Hz, 2H), 3.88 (s, 3H), 3.94 (s, 3H), 4.02 (s, 3H), 7.00 (d, J =1.55, 2H), 7.02 (s, 1H), 7.12 (s, 1H), 8.03 (d, J =8.9, 2H), 8.65 (s, 1H), (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.98 (CH_2, C-aliphatic), 46.01 (CH_3, C-aliphatic), 52.56 (CH_2, C-aliphatic), 55.22 (CH_3, C-aliphatic), 55.43 (CH_2, C-aliphatic), 55.78 (CH_2, C-aliphatic), 56.13 (CH_3, C-aliphatic), 56.42 (CH_3, C-aliphatic), 104.61 (CH, C-aromatic), 109.37 (CH, C-aromatic), 111.14 (C, C-aromatic), 113.98 (CH, C-aromatic), 127.27 (C, C-aromatic), 129.20 (CH, C-aromatic), 136.39 (C, C-aromatic), 143.95 (C, C-aromatic), 152.43 (C, C-aromatic), 162.43 (C, C-aromatic), 165.18 (C, C-aromatic), 168.87 (C, C-aromatic),

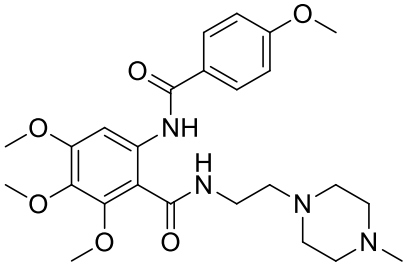
MS (ESI)$^+$: 457.3 $[M+H]^+$

m.p.: (from ethanol/water) 69-71 °C

Synthesis of 2, 3, 4-trimethoxy-6-(4-methoxybenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl)benzamide (178)

Chemical formula: $C_{25}H_{34}N_4O_6$

Molecular Weight: 486.25



Procedure: 2

Yield: 38 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.31 (s, 3H), 2.43-2.44 (m, 8H), 2.47 (t, J =5.7 Hz, 2H), 3.43 (q, J =5.3 Hz, 2H), 3.89 (s, 3H), 3.9 (s, 3H), 3.91 (s, 3H), 3.93 (s, 3H), 6.91 (s, 1H), 6.98 (d, J =8.5 Hz, 2H), 7.00-7.05 (m, 1H), 7.95 (d, J =8.5 Hz, 2H), 8.65 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.32 (CH_2, C-aliphatic), 45.71 (CH_3, C-aliphatic), 52.38 (CH_2, C-aliphatic), 54.77 (CH_3, C-aliphatic), 55.51 (CH_2, C-aliphatic), 56.23 (CH_2, C-aliphatic), 56.32 (CH_3, C-aliphatic), 61.00 (CH_3, C-aliphatic), 61.07 (CH_3, C-aliphatic), 106.43 (CH, C-aromatic), 113.95 (CH, C-aromatic), 122.68 (C, C-aromatic), 126.22 (C, C-aromatic), 127.65 (C, C-aromatic), 129.49 (CH, C-aromatic), 144.50 (C, C-aromatic), 149.18 (C, C-aromatic), 151.87 (C, C-aromatic), 162.72 (C, C-aromatic), 166.67 (C, C-aromatic), 167.83 (C, C-aromatic) ppm.

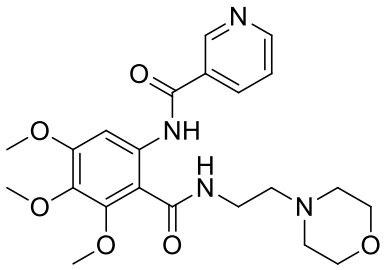
MS (ESI)$^+$: 487.3 $[M+H]^+$

m.p.: 96 – 98 °C

Synthesis of N-(3, 4, 5-trimethoxy-2-(2-morpholinoethylcarbamoyl) phenyl) nicotinamide (179)

Chemical formula: $C_{22}H_{28}N_4O_6$

Molecular Weight: 444.20



Procedure: 2

Yield: 31 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.48 (s, 4H), 2.56 (t, J = 5.6 Hz, 2H), 3.51-3.45 (m, 2H), 3.70-3.66 (m, 4H), 3.93 (s, 3H), 3.94 (d, J = 1.9 Hz, 6H), 6.9 (s, 1H), 6.98 (s, 1H), 7.45 (dd, J = 5.1, 7. 65 Hz, 1H), 8.28 (dt, J = 1.8, 7.95 Hz, 1H), 8.79 (d, J = 4.1 Hz, 1H), 9.09, (s, 1H), 9.3 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.86 (CH_2, C-aliphatic), 53.22 (CH_2, C-aliphatic), 56.38 (CH_3, C-aliphatic), 56.87 (CH_2, C-aliphatic), 61.00 (CH_3, C-aliphatic), 61.06 (CH_3, C-aliphatic), 66.58 (CH_2, C-aliphatic), 106.10 (CH, C-aromatic), 122.48 (C, C-aromatic), 123.52 (CH, C-aromatic), 129.81 (C, C-aromatic), 135.38 (CH, C-aromatic), 144.72 (C, C-aromatic), 148.90 (CH, C-aromatic), 149.26 (C, C-aromatic), 152.04 (C, C-aromatic), 152.68 (CH, C-aromatic), 159.51 (C, C-aromatic), 164.96 (C, C-aromatic), 167.95 (C, C-aromatic) ppm.

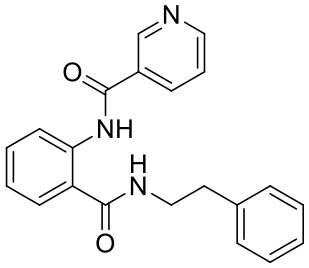
MS (ESI)$^+$: 445.2 $[M+H]^+$

m.p.: (from ethanol/water): 154-156 ˚C

Synthesis of N-(2-(phenethylcarbamoyl)phenyl)nicotinamide (180)

Chemical formula: $C_{21}H_{19}N_3O_2$

Molecular Weight: 345.15



Procedure: 2

Yield: 99 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.97 (t, J =10 Hz, 2H), 3.76 (q, J =10.0 Hz, 2H), 6.62 (s, 1H), 7.08 (t, J =9.97 Hz, 1H), 7.26 (q, J =10.02 Hz, 3H), 7.34 (t,J =9.90 Hz, 2H,), 7.40 (dd, J = 0.5, 9.96 Hz, 1H), 7.48 (dd, J =4.98, 9.92 Hz 1H), 7.52 (t, J =4.95 Hz, 1H), 8.31 (dt, J =10.2 Hz, 1H), 8.763-8.79 (m, 2H), 9.30 (s, 1H), 12.40 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.52 (CH_2, C-aliphatic), 41.17 (CH_2, C-aliphatic), 120.32 (C, C-aromatic), 121.54 (CH, C-aromatic), 123.29 (CH, C-aromatic), 123.59 (CH, C-aromatic), 126.51 (CH, C-aromatic), 126.79 (CH, C-aromatic), 128.81 (CH, C-aromatic), 130.51 (C, C-aromatic), 132.80 (CH, C-aromatic), 135.03 (CH, C-aromatic), 138.50 (C, C-aromatic), 139.60 (C, C-aromatic), 149.01 (CH, C-aromatic), 152.30 (CH, C-aromatic), 163.64 (C, C-aromatic), 169.12 (C, C-aromatic) ppm.

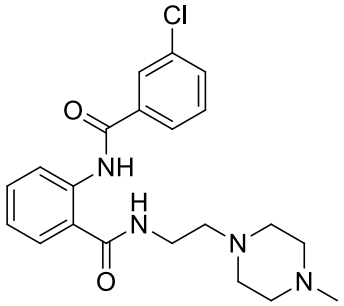
MS (ESI)$^+$: 346.2 $[M+1]^+$

m.p.: (from ethanol/water) 93-95 °C

Synthesis of 2-(4-chlorobenzamido)-N-(2-(4-methylpiperazin-1-yl)ethyl) benzamide (181)

Chemical formula: $C_{21}H_{25}ClN_4O_2$

Molecular Weight: 400.17



Procedure: 2

Yield: 64 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.35 (s, 3H), 2.42-2.66 (m, 8H), 2.68 (t, J =5.27 Hz, 2H), 3.57 (t, J =5.02 Hz, 2H), 7.17 (s, 1H), 7.20 (t, J =9.95 Hz, 1H), 7.47 (dd, J = 4.9, 9. 9 Hz, 1H), 7.55-7.62 (m, 2H), 8.33(dt, J =4.99, 10.00 Hz, 1H), 8.79 (dd, J =5.06, 9.98 Hz, 1H), 8.84 (d, J =10.03 Hz, 1H), 9.32 (d, J =5.02 Hz, 1H), 12.56 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.25 (CH_2, C-aliphatic), 45.94 (CH_3, C-aliphatic), 52.65 (CH_2, C-aliphatic), 55.09 (CH_2, C-aliphatic), 55.82 (CH_2, C-aliphatic), 120.15 (C, C-aromatic), 121.52 (CH, C-aromatic), 123.03 (CH, C-aromatic), 126.59 (CH, C-aromatic), 128.87 (CH, C-aromatic), 129.01 (CH, C-aromatic), 132.80 (CH, C-aromatic), 133.32 (C, C-aromatic), 138.08 (C, C-aromatic), 140.06 (C, C-aromatic), 164.46 (C, C-aromatic), 169.08 (C, C-aromatic) ppm.

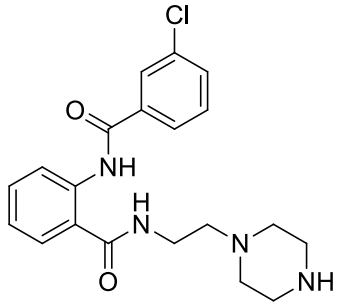
MS (ESI)$^{+}$: 401.2 $[M+H]^{+}$

m.p.: (from ethanol/water) 98-100 °C

Synthesis of 2-(4-chlorobenzamido)-N-(2-(piperazin-1-yl)ethyl)benzamide (182)

Chemical formula: $C_{20}H_{23}ClN_4O_2$

Molecular Weight: 386.15



Yield: 63 %

State: yellow solid

^{1}H-NMR ($CDCl_3$): δ 2.41-2.56 (m, 5H), 2.61 (t, J = 5.75 Hz, 2H), 2.91 (t, J = 4.2 Hz, 4H), 3.54 (q, J = 5.8 Hz, 2H), 7.13 (t, J = 7.35 Hz, 1H), 7.24-7.28 (m, 1H), 7.47 (d, J = 8. 4 Hz, 2H), 7.50-7.56 (m, 2H), 7.98 (d, J = 8.4 Hz, 2H), 8.78 (d, J = 8.4 Hz, 1H), 12.38 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.13 (CH_2, C-aliphatic), 45.96 (CH_2, C-aliphatic), 53.91 (CH_2, C-aliphatic), 56.58 (CH_2, C-aliphatic), 120.18 (C, C-aromatic), 121.44 (CH, C-aromatic), 123.04 (CH, C-aromatic), 126.71 (CH, C-aromatic), 128.85 (CH, C-aromatic), 129.00 (CH, C-aromatic), 132.73 (CH, C-aromatic), 133.29 (C, C-aromatic), 138.06 (C, C-aromatic), 139.96 (C, C-aromatic), 164.47 (C, C-aromatic), 169.10 (C, C-aromatic) ppm.

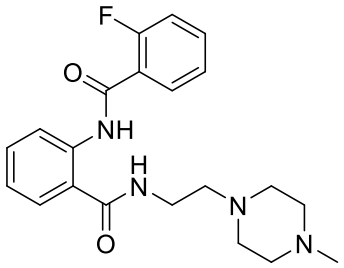
MS (ESI)$^+$: 387.2 $[M+H]^+$

m.p.: (from ethanol/water) 59-61 °C

Synthesis 2-fluoro-N-(2-((2-(4-methylpiperazin-1-yl)ethyl)carbamoyl)phenyl)benzamide (183)

Chemical Formula: $C_{21}H_{25}FN_4O_2$

Molecular Weight: 384.46



Procedure: 2

Yield: 25 %

State: white solid.

^{1}H-NMR ($CDCl_3$): δ 2.29 (s, 3H), 2.33-2.58 (m, 8H,), 2.60 (t, *J* =6.1 Hz, 2H), 3.51 (q, *J* = 4.5 Hz, 2H), 7.0 (s, 1H), 7.13 (td, *J* =1.05, 7.65 Hz, 1H), 7.18 (dd, *J* =0.9, 8.3 Hz, 1H), 7.26 (td, *J* =1, 7.55 Hz, 1H), 7.45-7.52 (m, 3H), 8.03 (td, *J* =1.85, 7.7 Hz, 1H), 8.73 (d, *J* =8.1 Hz, 1H), 11.83 (d, *J* =6.5 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.31 (CH_2, C-aliphatic), 45.94 (CH_3, C-aliphatic), 52.70 (CH_2, C-aliphatic), 55.11 (CH_2, C-aliphatic), 55.99 (CH_2, C-aliphatic), 116.52 (CH, C-aromatic), 121.96 (C, C-aromatic), 122.37 (CH, C-aromatic), 122.80 (CH, C-aromatic), 123.34 (CH, C-aromatic), 124.59 (CH, C-aromatic), 126.68 (CH, C-aromatic), 131.46 (CH, C-aromatic), 132.21 (CH, C-aromatic), 139.05 (C, C-aromatic), 159.3 (C, C-aromatic), 161.33 (C, C-aromatic), 162.28 (C-C-aromatic), 168.61 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.49 ppm.

MS(ESI)$^{+}$: 385.2 $[M+H]^{+}$

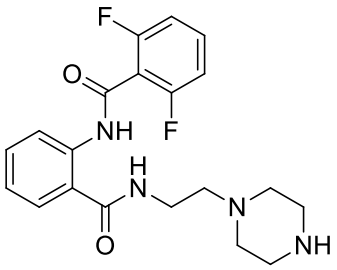
m.p.: (from ethanol/water) 115-117 ^{o}C

Accurate Mass calculated : 403.1940; **found:** 403.1936.

HPLC (method 1): retention time 10.01 minutes.

Synthesis of 2, 6-difluoro-N-(2-(2-(piperazin-1-yl)ethylcarbamoyl) phenyl) benzamide (184)
Chemical formula: $C_{20}H_{22}F_2N_4O_2$

Molecular Weight: 388.17



Procedure: 2

Yield: 34%

State: white solid

^{1}H-NMR ($CDCl_3$): δ 1.96 (s, 1H), 2.47 (s, 4H), 2.59 (t, J =5.2 Hz, 2H), 2.94-2.88 (m, 4H), 3.49 (q, J =5.2 Hz, 2H), 7.0 (t, J =7.25 Hz, 2H), 7.18-7.12 (m, 2H), 7.39 (q, J =7.25 Hz, 1H), 7.57-7.50 (m, 2H), 8.81 (d, J =7.25 Hz, 1H), 11.81 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 36.04 (CH_2, C-aliphatic), 46.16 (CH_2, C-aliphatic), 54.12 (CH_2, C-aliphatic), 56.52 (CH_2, C-aliphatic), 112.07 (CH, C-aromatic), 112.24 (CH, C-aromatic), 120.69 (C, C-aromatic), 121.75 (CH, C-aromatic), 123.57 (CH, C-aromatic), 126.58 (CH, C-aromatic), 131.78 (CH, C-aromatic), 132.64 (CH, C-aromatic), 139.15 (C, C-aromatic), 158.93 (C, C-aromatic), 160.97 (C, C-aromatic), 168.63 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 112.32 ppm.

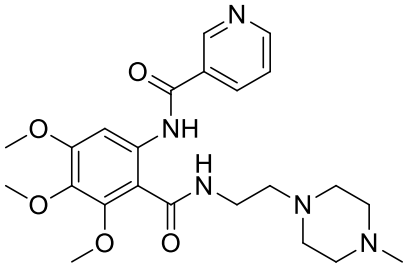
MS (ESI)$^+$: 389.2 $[M+H]^+$

m.p.: (from ethanol/water) 78-80 ˚C

Synthesis of N-(3,4,5-trimethoxy-2-(2-(4-methylpiperazin-1-yl) ethylcarbamoyl) phenyl)nicotinamide (185)

Chemical formula: $C_{23}H_{31}N_5O_5$

Molecular Weight: 457.23



Procedure: 2

Yield: 47 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.32 (s, 3H), 2.55-2.34 (m, 8H), 2.58 (t, J = 6 Hz, H), 3.47 (q, J = 5.7 Hz, 2H), 3.93 (s, 3H), 3.94 (s, 3H), 3.95 (s, 3H), 6.9 (s, 1H), 7.02 (s, 1H), 7.45 (dd, J = 4.8, 7.8 Hz, 1H, $H_{6'}$), 8.28 (td, J = 1.8, 8.1 Hz, 1H, $H_{4'}$), 8.8 (dd, J = 1.5, 5.2 Hz, 1H), 9.2 (s, 1H), 9.22 (d, J = 2.1 Hz, 1H) ppm.

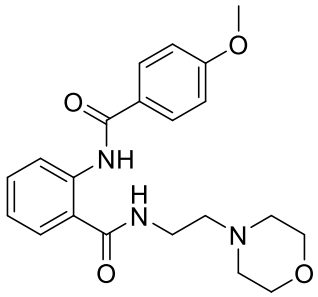
^{13}C-NMR ($CDCl_3$): δ 36.15 (CH_2, C-aliphatic), 45.70 (CH_3, C-aliphatic), 52.38 (CH_2, C-aliphatic), 54.65 (CH_2, C-aliphatic), 56.17 (CH_2, C-aliphatic), 56.39 (CH_3, C-aliphatic), 61.01 (CH_3, C-aliphatic), 61.04 (CH_3, C-aliphatic), 106.06 (CH, C-aromatic), 122.68 (C, C-aromatic), 123.53 (CH, C-aromatic), 126.40 (C, C-aromatic), 129.84 (C, C-aromatic), 135.37 (CH, C-aromatic), 144.77 (C, C-aromatic), 148.94 (CH, C-aromatic), 149.30 (C, C-aromatic), 151.98 (C, C-aromatic), 152.66 (CH, C-aromatic), 164.90 (C, C-aromatic), 167.89 (C, C-aromatic) ppm.

MS (ESI)$^+$: 458.3 $[M+H]^+$

m.p.: (from ethanol/water) 142-144 °C

Synthesis of 2-(4-methoxybenzamido)-*N*-(2-morpholinoethyl)benzamide (186)
Chemical Formula: $C_{21}H_{25}N_3O_4$

Molecular Weight: 383.45



Procedure: 2

Yield: 81%

State: white solid

^{1}H-NMR ($CDCl_3$): δ 2.45 (s, 4H), 2.56 (t, *J* = 5.0 Hz, 2H), 3.48 (dd, *J* = 5.5, 11.2 Hz, 2H), 3.61 – 3.73 (m, 4H), 3.80 (s, 3H), 6.81 – 6.98 (m, 3H), 7.04 (tt, *J* = 13.3, 26.6 Hz, 1H), 7.41 – 7.51 (m, 2H), 7.89 – 7.98 (m, 2H), 8.74 (dd, *J* = 0.8, 8.4 Hz, 1H), 12.06 (s, 1H) ppm.

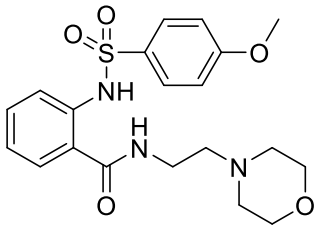
^{13}C-NMR ($CDCl_3$): δ 35.97 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 55.47 (CH_3, C-aliphatic), 56.49 (CH_2, C-aliphatic), 66.99 (CH_2, C-aliphatic), 113.97 (CH, C-aromatic), 120.22 (C, C-aromatic), 121.58 (CH, C-aromatic), 122.63 (CH, C-aromatic), 126.52 (CH, C-aromatic), 127.21 (C, C-aromatic), 129.32 (CH, C-aromatic), 132.76 (CH, C-aromatic), 140.33 (C, C-aromatic), 140.33 (C, C-aromatic), 162.48 (C, C-aromatic), 165.18 (C, C-aromatic), 169.24 (C, C-aromatic) ppm.

MS (ESI)$^+$: 384.2$[M+H]^+$

m.p.: 89-91 °C

Synthesis of 2-((4-methoxyphenyl)sulfonamido)-*N*-(2-morpholinoethyl)benzamide (187)
Chemical Formula: $C_{20}H_{25}N_3O_5S$

Molecular Weight: 419.50



Procedure: 5

Yield: 26 %

State: orange powder

^{1}H-NMR (DMSO-d$_6$): δ 2.36 – 2.43 (m, 4H), 2.47 (t, J = 6.0 Hz, 2H), 3.29 – 3.43 (m, 2H), 3.57 – 3.66 (m, 4H), 3.73 (s, 3H), 6.67 (t, J = 13.8 Hz, 1H), 6.74 – 6. 80 (m, 2H), 6.98 (dd, J = 5.1, 10.9 Hz, 1H), 7.26 – 7.34 (m, 2H), 7.58 (dd, J = 0.9, 8.3 Hz, 1H), 7.65 – 7.74 (m, 2H), 11.2 (s, 1H) ppm.

^{13}C-NMR (DMSO-d$_6$): δ 35.88 (CH_2, C-aliphatic), 53.25 (CH_2, C-aliphatic), 55.55 (CH_3, C-aliphatic), 56.46 (CH_2, C-aliphatic), 67.26 (CH_2, C-aliphatic), 114.02 (CH, C-aromatic), 121.04 (CH, C-aromatic), 121.25 (C, C-aromatic), 123.35 (CH, C-aromatic), 126.68 (CH, C-aromatic), 129.42 (CH, C-aromatic), 131.38 (C, C-aromatic), 132.53 (CH, C-aromatic), 139.09 (C, C-aromatic), 162.90 (C, C-aromatic), 168.21 (C, C-aromatic) ppm.

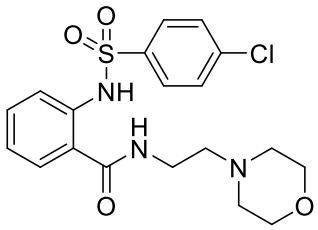
MS (ESI)$^-$: 418.2 $[M - H]^-$

m.p: 70 – 72 °C

Synthesis of 2-((4-chlorophenyl)sulfonamido)-N-(2-morpholinoethyl)benzamide (188)

Chemical Formula: $C_{19}H_{22}ClN_3O_4S$

Molecular Weight: 423.91



Procedure: 5

Yield: 61 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.39 – 2.43 (m, 4H), 2.48 (t, J = 6.0 Hz, 1H), 2.99 (s, 1H), 3.35 (dd, J = 5.8, 10.7 Hz, 2H), 3.62 – 3.65 (m, 4H), 6.77 (s, 1H), 7.03 (td, J = 1.1, 7.8 Hz, 1H), 7.28 – 7.30 (m, 1H), 7.32 – 7.36 (m, 1H), 7.59 (dd, J = 0.9, 8.3, Hz, 4H), 7.65 – 7.68 (m, 1H), 11.05 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 29.70 (CH_2, C-aliphatic), 36.04 (CH_2, C-aliphatic), 56.42 (CH_2, C-aliphatic), 66.95 (CH_2, C-aliphatic), 121.46 (C, C-aromatic), 121.53 (CH, C-aromatic), 123.69 (CH, C-aromatic), 126.75 (CH, C-aromatic), 128.62 (CH, C-aromatic), 128.70 (CH, C-aromatic), 129.12 (CH, C-aromatic), 132.84 (CH, C-aromatic), 132.90 (CH, C-aromatic), 138.19 (C, C-aromatic), 138.67 (C, C-aromatic), 139.13 (C, C-aromatic), 168.06 (C, C-aromatic) ppm.

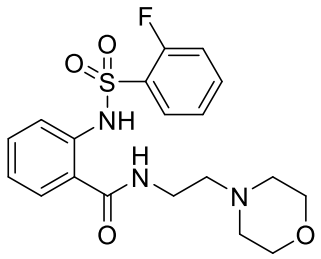
MS (ESI)$^-$: 418.2 $[M - H]^-$

m.p.: 62 – 64 °C

Synthesis of 2-((2-fluorophenyl)sulfonamido)-N-(2-morpholinoethyl)benzamide (189)

Chemical Formula: $C_{19}H_{22}FN_3O_4S$

Molecular Weight: 407.46



Procedure: 5

Yield: 51 %

State: colourless oil

^{1}H-NMR ($CDCl_3$): δ 2.37 – 2.44 (m, 3H), 2.47 – 2.55 (m, 1H), 2.93 (s, 2H), 3.39 – 3.48 (m, 2H), 3.56 – 3.63 (m, 2H), 6.44 (s, 2H), 6.85 (dt, J = 6.3, 12.9 Hz, 1H), 6.91 – 7.02 (m, 1H), 7.05 –7.18 (m, 2H), 7.29 – 7.39 (m, 1H), 7.42 (t, J = 7.6 Hz, 1H), 7.59 (dd, J = 3.54, 10.3 Hz, 1H), 7.80 – 7.90 (m, 1H), 8.10 (s, 1H), 11.18 (1s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 40.03 (CH_2, C-aliphatic), 54.50 (CH_2, C-aliphatic), 55.92 (CH_2, C-aliphatic), 64.91 (CH_2, C-aliphatic), 114.9 (CH, C-aromatic), 117.10 (CH, C-aromatic), 117.82 (C, C-aromatic), 118.4 (CH, C-aromatic), 119.1 (CH, C-aromatic), 124.2 (CH, C-aromatic), 126.2 (C, C-aromatic), 127.9 (CH, C-aromatic), 130.10 (CH, C-aromatic), 132.00 (CH, C-aromatic), 146.10 (C, C-aromatic), 158.9 (C, C-aromatic), 169.7 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): -108.86 ppm.

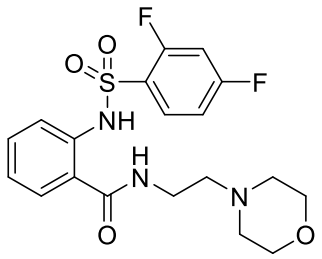
MS (ESI)$^+$: 408.14 $[M + H]^+$

m.p.:-

Synthesis of 2-((2, 4-difluorophenyl)sulfonamido)-N-(2-morpholinoethyl)benzamide (190)

Chemical Formula: $C_{19}H_{21}F_2N_3O_4S$

Molecular Weight: 425.45



Procedure: 5

Yield: 58%

State: colourless oil

^{1}H-NMR ($CDCl_3$): δ 2.36 – 2.46 (m, 4H), 2.52 (t, J = 6.0 Hz, 2H), 3.40 – 3.48 (m, 2H), 3.62 – 3.68 (m, 4H), 6.77 (dd, J = 2.4, 8.5 Hz, 2H), 6.84 – 6.94 (m, 1H), 7.00 (td, J = 1.1, 7.7 Hz, 1H), 7.25 – 7.38 (m, 1H), 7.47 – 7.54 (m, 1H), 7.85 – 7.96 (m, 1H), 8.78 – 8.86 (m, 1H), 11.3 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.94 (CH_2, C-aliphatic), 53.29 (CH_2, C-aliphatic), 56.49 (CH_2, C-aliphatic), 66.96 (CH_2, C-aliphatic), 105.66 (CH, C-aromatic, t, J = 25 Hz), 111.77 (CH, C-aromatic, d, J = 25 Hz), 119.39 (CH, C-aromatic), 120.49 (C, C-aromatic), 123.30 (CH, C-aromatic), 126.87 (CH, C-aromatic), 132.52 (CH, C-aromatic), 132.90 (CH, C-aromatic), 138.45 (C, C-aromatic), 158.81 (C, C-aromatic, d, J = 12.5 Hz), 160.87 (C, C-aromatic, d, J = 8.75 Hz), 166.89 (C, C-aromatic, d, J = 12.5 Hz), 168.14 (C, C-aromatic) ppm

^{19}F-NMR ($CDCl_3$): δ -100.20, -107.88 ppm

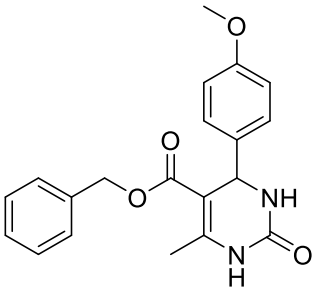
MS (ESI)$^-$: 424.1 $[M - H]^-$

m.p.: -

Synthesis of benzyl 4-(4-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (191)

Chemical Formula: $C_{20}H_{20}N_2O_4$

Molecular Weight: 352.39



Procedure: 6

Yield: 78%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.38 (s, 3H), 3.81 (s, 3H), 4.95 (d, *J* =12.4 Hz, 1H), 5.04 (d, *J* =12.4 Hz, 1H), 5.38 (d, *J* =2.65 Hz, 1H), 5.58 (s, 1H), 6.81-6.83 (d, *J* =8.6 Hz, 2H), 7.14 – 7.20 (m, 4H), 7.31 (t, *J* = 3.15 Hz, 3H), 7.70 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.78 (CH_3, C-aliphatic), 55.25 (CH, C-aliphatic), 55.30 (CH_3, C-aliphatic), 65.97 (CH_2, C-aliphatic), 101.55 (C, C-aromatic), 114.12 (CH, C-aromatic), 127.90 (CH, C-aromatic), 127.98 (CH, C-aromatic), 128.04 (CH, C-aromatic), 128.06 (CH, C-aromatic), 128.43 (CH, C-aromatic), 130.21 (CH, C-aromatic), 135.66 (C, C-aromatic), 135.95 (C, C-aromatic), 146.22 (C, C-aromatic), 152.90 (C, C-aromatic), 159.41 (C, C-aromatic), 165.25 (C, C-aromatic) ppm.

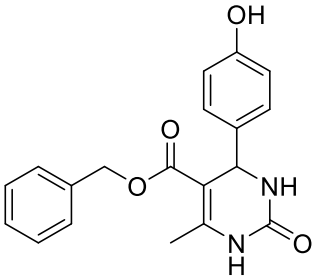
MS (ESI)$^+$: 353.1 $[M + H]^+$

m.p: (from ethanol/water) 167 - 170 °C

Synthesis of benzyl 4-(4-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (192)

Chemical Formula: $C_{19}H_{18}N_2O_4$

Molecular Weight: 338.36



Procedure: 6

Yield: 24%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.28 (s, 3H), 4.96 – 5.15 (m, 4H), 6.65 – 6.72 (m, 2H), 7.02 (t, J = 11.3 Hz, 2H), 7.17 (dd, J = 2.1, 7.2 Hz, 2H), 7.24 – 7.35 (m, 2H), 7.62 (s, 1H), 9.16 (s, 1H), 9.32 (s, 1H) ppm.

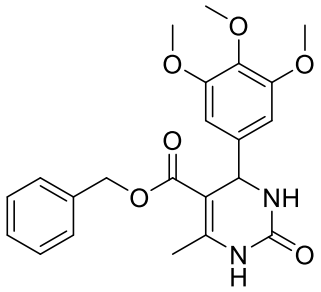
^{13}C-NMR ($CDCl_3$): δ 18.78 (CH_3, C-aliphatic), 55.25 (CH, C-aliphatic), 65.97 (CH_2, C-aliphatic), 101.55 (C, C-aromatic), 114.12 (CH, C-aromatic), 127.90 (CH, C-aromatic), 128.02 (CH, C-aromatic), 128.06 (CH, C-aromatic), 128.43 (CH, C-aromatic), 135.66 (C, C-aromatic), 135.95 (C, C-aromatic), 146.22 (C, C-aromatic), 159.41 (C, C-aromatic), 165.26 (C, C-aromatic) ppm.

MS(ESI)$^+$: 353.1 $[M + H]^+$

m.p: (from ethanol/water) 134- 136 °C

Synthesis of benzyl 6-methyl-2-oxo-4-(3, 4, 5-trimethoxyphenyl)-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (193)

Chemical Formula: $C_{22}H_{24}N_2O_6$
Molecular Weight: 412.44



Procedure: 6

Yield: 28%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.32 (s, 3H), 3.01 (s, 3H), 3.64 (s, 3H), 4.95 (d, *J* =12.46 Hz, 1H), 5.04 (d, *J* =12.4 Hz, 1H), 5.11 (d, *J* =12.4 Hz, 1H), 5.30 (d, *J* =2.51 Hz, 1H), 5.58 (s, 1H), 6.38 (s, 2H), 7.05 – 7.09 (m, 2H), 7.20 – 7.24 (m, 3H), 7.85 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.86 (CH_3, C-aliphatic), 56.01 (CH_3 and CH, C-aliphatic), 60.85 (CH_3, C-aliphatic), 66.08 (CH_2, C-aliphatic), 100.75 (C, C-aromatic), 103.51 (CH, C-aromatic), 128.01 (CH, C-aromatic), 128.16 (CH, C-aromatic), 128.49 (CH, C-aromatic), 135.87 (C, C-aromatic), 137.67 (C, C-aromatic), 139.20 (C, C-aromatic), 147.00 (C, C-aromatic), 152.84 (C, C-aromatic), 153.41 (C, C-aromatic), 165.39 (C, C-aromatic) ppm.

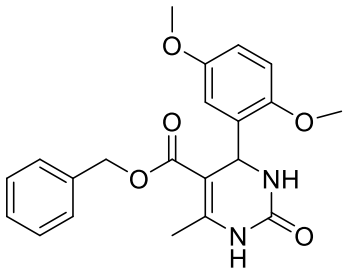
MS(ESI)$^+$: 413.2 $[M + H]^+$

m.p: (from ethanol/water) 134 – 136 °C

Synthesis of benzyl 4-(2, 5-dimethoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (194)

Chemical Formula: $C_{21}H_{22}N_2O_5$

Molecular Weight: 382.42



Procedure: 6

Yield: 85%

State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 2.45 (s, 3H), 3.70 (s, 3H), 3.80 (s, 3H), 5.03 (d, *J* =12.77 Hz,1H), 5.11 (d, *J* =12.78 Hz, 1H), 5.77 (d, *J* =2.76, 1H), 5.80 (d, *J* =2.02 Hz, 1H), 6.66 (d, *J* = 2.97 Hz, 1H), 6.78 (dd, *J* =2.98, 8.83 Hz, 1H), 6.83 (d, *J* =8.83 Hz, 1H), 7.04 – 7.06 (m, 2H) 7.23 – 7.26 (m, 3M), 7.98 (s, 1H) ppm.

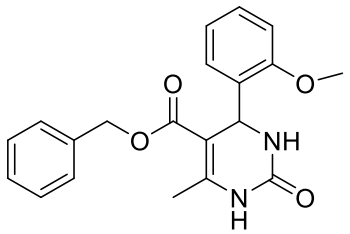
^{13}C-NMR ($CDCl_3$): δ 18.72 (CH_3, C-aliphatic), 49.84 (CH, C-aliphatic), 55.65 (CH_3, C-aliphatic), 55.73 (CH_3, C-aliphatic), 65.57 (CH_2, C-aliphatic), 97.80 (C, C-aromatic), 111.27 (CH, C-aromatic), 112.34 (CH, C-aromatic), 113.72 (CH, C-aromatic), 127.44 (CH, C-aromatic), 127.73 (CH, C-aromatic), 128.31 (C, C-aromatic), 130.91 (C, C-aromatic), 136.28 (C, C-aromatic), 149.15 (C, C-aromatic), 150.89 (C, C-aromatic), 153.23 (C, C-aromatic), 153.63 (C, C-aromatic), 165.39 (C, C-aromatic) ppm.

MS(ESI)$^+$: 405.1 $[M + Na]^+$

m.p: (from ethanol/water) 122 - 124 °C

Synthesis of benzyl 4-(2-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (195)

Chemical Formula: $C_{20}H_{20}N_2O_4$
Molecular Weight: 352.39



Procedure: 6

Yield: 89%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.46 (s, 3H), 3.85 (s, 3H), 5.02 (d, *J* =12.81 Hz, 1H), 5.09 (d, *J* =12.80 Hz, 1H), 5.82 (dd, *J* = 4.34, 8.62 Hz, 1H), 5.83 (d, *J* =6.07 Hz, 1H), 6.90 (dd, *J* =5.53, 9.38 Hz, 2H), 7.02 (td, *J* =2.44, 4.79 Hz, 2H), 7.08 (dd, *J* =1.47, 7.52 Hz, 1H), 7.21 – 7.30 (m, 4H), 8.36 (s, 1H) ppm.

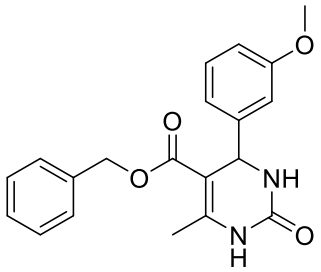
^{13}C-NMR ($CDCl_3$): δ 18.60 (CH_3, C-aliphatic), 49.78 (CH, C-aliphatic), 55.32 (CH_3, C-aliphatic), 65.52 (CH_2, C-aliphatic), 97.88 (C, C-aromatic), 110.55 (CH, C-aromatic), 120.73 (CH, C-aromatic), 126.63 (CH, C-aromatic), 127.39 (CH, C-aromatic), 127.70 (CH, C-aromatic), 128.29 (CH, C-aromatic), 128.54 (CH, C-aromatic), 129.16 (CH, C-aromatic), 129.74 (C, C-aromatic), 136.29 (C, C-aromatic), 149.16 (C, C-aromatic), 153.55 (C, C-aromatic), 156.70 (C, , C-aromatic), 165.53 (C, , C-aromatic) ppm.

MS(ESI)$^+$: 353.1 $[M + H]^+$

m.p: (from ethanol/water) 140 - 142 °C

Synthesis of benzyl 4-(3-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (196)

Chemical Formula: $C_{20}H_{20}N_2O_4$
Molecular Weight: 352.39



Procedure: 6

Yield: 65%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.37 (s, 3H), 3.74 (s, 3H), 5.06 (d, *J* =12.50 Hz, 1H), 5.11 (d, *J* =12.54 Hz, 1H), 5.41 (d, *J* =1.96 Hz, 1H), 5.66 (d, *J* =5.62 Hz, 1H), 6.82 – 6.89 (m, 3H), 7.15 – 7.25 (m, 3H), 7.30 – 7.34 (m, 3H), 7.97 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.72 (CH_3, C-aliphatic), 55.16 (CH_3, C-aliphatic), 55.64 (CH, C-aliphatic), 65.92 (CH_2, C-aliphatic), 97.88 (C, C-aromatic), 112.47 (CH, C-aromatic), 113.22 (CH, C-aromatic), 118.85 (CH, C-aromatic), 127.99(CH, C-aromatic), 128.42 (CH, C-aromatic), 129.87 (CH, C-aromatic), 136.29 (C, C-aromatic), 145.10 (C, C-aromatic), 147.32 (C, C-aromatic), 153.24 (C, C-aromatic), 159.90 (C, C-aromatic), 165.36 (C, C-aromatic) ppm.

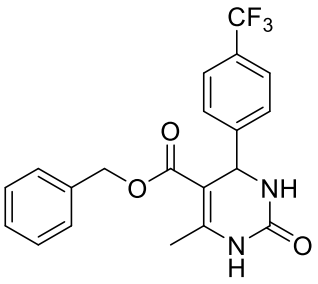
MS(ESI)$^+$: 353.2 [M + H^+]

m.p: (from ethanol/water) 84 - 86 °C

Synthesis of benzyl 6-methyl-2-oxo-4-(4-(trifluoromethyl)phenyl)-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (197)

Chemical Formula: $C_{20}H_{17}F_3N_2O_3$

Molecular Weight: 390.36



Procedure: 6

Yield: 92%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.40 (s, 3H), 5.02 (d, *J* =12.31 Hz, 1H), 5.15 (d, *J* =12.30 Hz, 1H), 5.48 (d, *J* =2.89 Hz, 1H), 5.80 (s, 1H), 7.13 – 7.14 (m, 2H), 7.29 – 7.33 (m, 3H), 7.38 (d, *J* =8.08, 2H), 7.53 – 7.54 (m, 2H), 7.92 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.87 (CH_3, C-aliphatic), 55.41 (CH, C-aliphatic), 66.14 (CH_2, C-aliphatic), 100.43 (C, C-aromatic), 125.79 (CH, C-aromatic), 127.05 (CH, C-aromatic), 128.18 (CH, C-aromatic), 128.25 (CH, C-aromatic), 128.49 (CH, C-aromatic), 130.12 (CH, C-aromatic), 130.37 (CH, C-aromatic), 135.70 (C, C-aromatic), 147.22 (C, C-aromatic), 147.46 (C, C-aromatic), 152.72 (C, C-aromatic), 164.98 (C, C-aromatic) ppm.

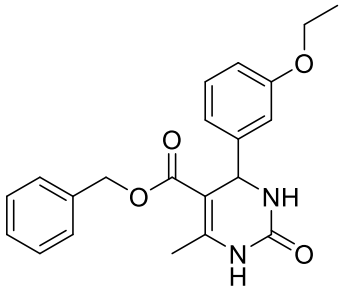
^{19}F-NMR ($CDCl_3$): δ -62.83 ppm

MS(ESI)$^+$: 391.2 $[M + H]^+$

m.p: (from Ethyl Acetate/*n*-hexane) 157 – 159 °C

Synthesis of benzyl 4-(3-ethoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (198)

Chemical Formula: $C_{21}H_{22}N_2O_4$
Molecular Weight: 366.42



Procedure: 6

Yield: 79%

State: pale beige powder

^{1}H-NMR ($CDCl_3$): δ 1.39 (s, 3H), 2.38 (s, 3H), 3.90 – 3.97 (m, 2H), 5.05 (d, *J* = 12.48 Hz, 1H), 5.11 (d, *J* = 12.49 Hz, 1H), 5.39 (d, *J* = 2.44 Hz, 1H), 5.78 (s, 1H), 6.80 – 6.89 (m, 3H), 7.15 – 7.23 (m, 3H), 7.30 (dd, *J* = 2.00, 4.44 Hz, 3H) 8.12 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 14.79 (CH_3, C-aliphatic), 18.76 (CH_3, C-aliphatic), 55.71 (CH, C-aromatic), 63.34 (CH_2, C-aliphatic), 65.92 (CH_2, C-aliphatic), 100.71 (C, C-aromatic), 113.04 (CH, C-aromatic), 113.74 (CH, C-aromatic), 118.76 (CH, C-aromatic), 127.96 (CH, C-aromatic), 127.99 (CH, C-aromatic), 128.41 (CH, C-aromatic), 128.83 (CH, C-aromatic), 136.03 (C, C-aromatic), 145.04 (C, C-aromatic), 147.22 (C, C-aromatic), 153.06 (C, C-aromatic), 159.29 (C, C-aromatic), 165.35 (C, C-aromatic) ppm.

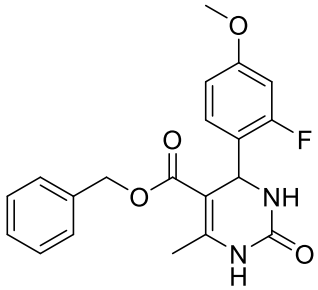
MS(ESI)$^+$: 367.2 $[M + H]^+$

m.p: (from ethanol/water) 98 - 100 °C

Synthesis of benzyl 4-(2-fluoro-4-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (199)

Chemical Formula: $C_{20}H_{19}FN_2O_4$

Molecular Weight: 370.38



Procedure: 6

Yield: 29 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.43 (s, 3H), 3.80 (s, 3H), 5.03 (d, J = 12.5 Hz, 1H), 5.09 (t, J = 14.2 Hz, 1H), 5.52 (s, 1H), 5.71 (d, J = 2.5 Hz, 1H), 6.60 (dt, J = 2.5, 8.9 Hz, 2H), 7.07 - 7.12 (m, 3H), 7.24 –7.30 (m, 3H), 7.93 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.71 (CH_3, C-aliphatic), 55.60 (CH, C-aliphatic), 55.69 (CH_3, C-aliphatic), 65.75 (CH_2, C-aliphatic), 98.56 (C, C-aromatic), 101.76 (CH, C-aromatic), 110.03 (CH, C-aromatic), 121.91 (C, C-aromatic), 127.79 (CH, C-aromatic), 127.91 (CH, C-aromatic), 128.36 (CH, C-aromatic), 128.73 (CH, C-aromatic), 136.06 (C, C-aromatic), 148.21 (C, C-aromatic), 159.95 (C, C-aromatic), 160.75 (C, C-aromatic), 161.91 (C, C-aromatic), 165.10 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -117.77 ppm.

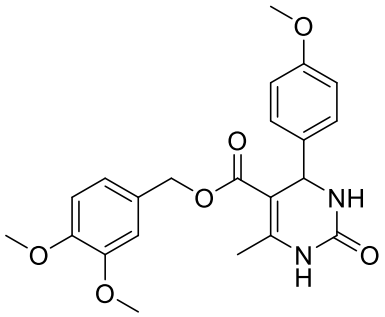
MS(ESI)$^+$: 371.1 $[M + H]^+$

m.p: (from hot ethanol) 145 - 147 °C

Synthesis of 3, 4-dimethoxybenzyl 4-(4-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (200)

Chemical Formula: $C_{22}H_{24}N_2O_6$

Molecular Weight: 412.44



Procedure: 6

Yield: 45%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.35 (s, 3H), 3.79 (s, 3H), 3.80 (s, 3H), 3.89 (s, 3H), 4.96 (d, *J* = 12.09 Hz, 1H), 5.05 (d, *J* = 12.09 Hz, 1H), 5.35 (d, *J* = 2.70 Hz, 1H), 5.81 (s, 1H), 6.70 (d, *J* = 1.81, 1H), 6.76 – 6.81 (m, 4H), 7.16 – 7.18 (m, 2H), 8.23 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.69 (CH_3, C-aliphatic), 55.12 (CH, C-aromatic), 55.24 (CH_3, C-aliphatic), 55.84 (CH_3, C-aliphatic), 55.91 (CH_3, C-aliphatic), 65.97 (CH_2, C-aliphatic), 101.24 (C, C-aromatic), 110.95 (CH, C-aromatic), 111.67 (CH, C-aromatic), 113.99 (CH, C-aromatic), 121.07 (CH, C-aromatic), 127.84 (CH, C-aromatic), 128.58 (C, C-aromatic), 135.99 (C, C-aromatic), 146.63 (C, C-aromatic), 148.92 (C, C-aromatic), 148.97 (C, C-aromatic), 153.26 (C, C-aromatic), 159.25 (C, C-aromatic), 165.50 (C, C-aromatic) ppm.

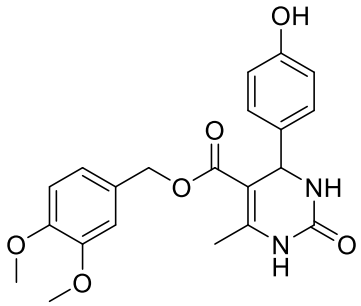
MS(ESI)$^+$: 413.2 $[M + H]^+$

m.p: (from ethanol/water) 157 - 159 °C

Synthesis of 3, 4-dimethoxybenzyl 4-(4-hydroxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (201)

Chemical Formula: $C_{21}H_{22}N_2O_6$

Molecular Weight: 398.42



Procedure: 6

Yield: 88%

State: white powder

^{1}H-NMR (DMSO-d$_6$): δ 2.25 (s, 3H), 3.68 (s, 3H), 3.74 (s, 3H), 4.94 (d, *J* =12.20 Hz, 1H), 4.97 (d, *J* =12.30 Hz, 1H), 5.07 (d, *J* =3.25 Hz, 1H), 6.67 (d, *J* =8.51 Hz, 2H), 6.76 – 6.77 (m, 1H), 6.81 (s, 1H), 6.88 (d, *J* =8.21, 1H), 7.00 (d, *J* =8.48 Hz, 2H) 7.60 (s, 1H), 9.13 (s, 1H), 9.33 (s, 1H) ppm.

^{13}C-NMR (DMSO-d$_6$): δ 17.76 (CH_3, C-aliphatic), 53.26 (CH, C-aliphatic), 55.36 (CH_3, C-aliphatic), 55.47 (CH_3, C-aliphatic), 64.91 (CH_2, C-aliphatic), 99.40 (C, C-aromatic), 111.50 (CH, C-aromatic), 111.73 (CH, C-aromatic), 114.95 (CH, C-aromatic), 120.42 (CH, C-aromatic), 127.35 (CH, C-aromatic), 128.76 (C, C-aromatic), 135.21 (C, C-aromatic), 148.41 (C, C-aromatic), 148.47 (C, C-aromatic), 148.53 (C, C-aromatic), 152.11 (C, C-aromatic), 156.54 (C, C-aromatic), 165.23 (C, C-aromatic) ppm.

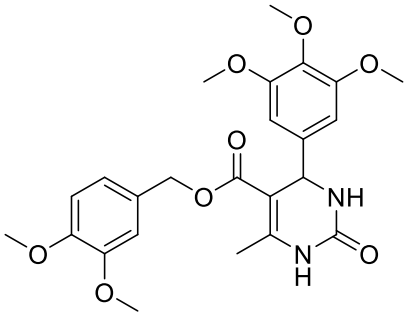
MS(ESI)$^+$: 399.2 $[M + H]^+$

m.p: (from ethanol/water) <200 °C

Synthesis of 3, 4-dimethoxybenzyl 6-methyl-2-oxo-4-(3, 4, 5-trimethoxyphenyl)-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (202)

Chemical Formula: $C_{24}H_{28}N_2O_8$

Molecular Weight: 472.49



Procedure: 6

Yield: 26%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.37 – 2.39 (s, 3H), 3.74 (s, 6H), 3.83 (s, 6H), 3.89 (s, 3H), 5.00 (d, *J* = 12.10 Hz, 1H), 5.06 (d, *J* = 12.20 Hz, 1H), 5.38 (d, *J* = 0.76 Hz, 1H), 5.72 (d, *J* = 0.54 Hz, 1H), 6.74 – 6.81 (m, 4H), 7.29 (s, 1H), 8.02 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.74 (CH_3, C-aliphatic), 55.84 (CH_3, C-aliphatic), 55.89 (CH_3, C-aliphatic), 55.92 (CH_3, C-aliphatic), 56.04 (CH_3, C-aliphatic), 56.15 (CH_3, C-aliphatic), 60.76 (CH, C-aliphatic), 66.14 (CH_2, C-aliphatic), 100.87 (C, C-aromatic), 103.66 (CH, C-aromatic), 111.03 (CH, C-aromatic), 111.68 (CH, C-aromatic), 121.00 (CH, C-aromatic), 128.43 (C, C-aromatic), 137.78 (C, C-aromatic), 139.19 (C, C-aromatic), 146.90 (C, C-aromatic), 148.95 (C, C-aromatic), 149.04 (C, C-aromatic), 153.15 (C, C-aromatic), 153.39 (C, C-aromatic), 165.50 (C, C-aromatic) ppm.

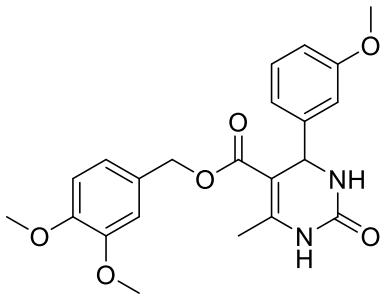
MS(ESI)$^+$: 495.2 $[M + Na]^+$

m.p: (from hot ethanol) 100 - 102 °C

Synthesis of 3, 4-dimethoxybenzyl 4-(3-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (203)

Chemical Formula: $C_{22}H_{24}N_2O_6$

Molecular Weight: 412.44



Procedure: 6

Yield: 73%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.37 (d, J = 6.9 Hz, 3H), 3.72 (s, 3H), 3.81 (s, 3H), 3.90 (s, 3H), 4.74 (s, 1H), 4.98 (d, J = 12.1 Hz, 1H), 5.04 (t, J = 13.2 Hz, 1H), 5.38 (d, J = 2.6 Hz, 1H), 5.67 (s, 1H), 6.71 (d, J = 1.6 Hz, 1H), 6.69-6.80 (m, 3H), 6.86 (d, J = 7.7 Hz, 1H), 7.16 – 7.24 (m, 1H), 7.78 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.72 (CH_3, C-aliphatic), 55.14 (CH_3, C-aliphatic), 55.63 (CH_3, C-aliphatic), 55.91 (CH_3, C-aliphatic), 65.95 (CH, C-aliphatic), 66.08 (CH_2, C-aliphatic), 100.91 (C, C-aromatic), 110.97 (CH, C-aromatic), 111.64 (CH, C-aromatic), 112.62 (CH, C-aromatic), 112.99 (CH, C-aromatic), 121.07 (CH, C-aromatic), 129.87 (CH, C-aromatic), 128.49 (C, C-aromatic), 129.83 (CH, C-aromatic), 145.06 (C, C-aromatic), 146.90 (C, C-aromatic), 148.99 (C, C-aromatic), 152.89 (C, C-aromatic), 159.85 (C, C-aromatic), 165.42 (C, C-aromatic) ppm.

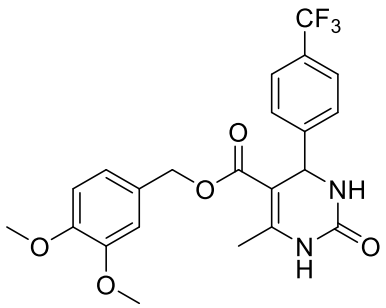
MS(ESI)$^+$: 413.2 $[M + H]^+$

m.p: (from ethanol/water) 160 - 162 °C

Synthesis of 3, 4-dimethoxybenzyl 6-methyl-2-oxo-4-(4-(trifluoromethyl)phenyl)-1,2,3,4-tetrahydropyrimidine-5-carboxylate (204)

Chemical Formula: $C_{22}H_{21}F_3N_2O_5$

Molecular Weight: 450.41



Procedure: 6

Yield: 38%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.38 (s, 3H), 3.81 (s, 3H), 3.89 (s, 3H), 4.96 (d, J = 11.99 Hz, 1H), 5.06 (d, J = 12.01 Hz, 1H), 5.46 (d, J = 2.78 Hz, 1H), 5.94 (s, 1H), 6.72 (dt, J = 2.07, 4.20 Hz, 2H), 6.79 – 6.80 (m, 1H), 7.37 (d, J = 8.10 Hz 2H), 7.53 (d, J = 8.24 Hz, 2H), 8.09 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.81 (CH_3, C-aliphatic), 55.31 (CH, C-aliphatic), 55.85 (CH_3, C-aliphatic), 55.88 (CH_3, C-aliphatic), 66.26 (CH_2, C-aliphatic), 100.51 (C, C-aromatic), 110.99 (CH, C-aromatic), 111.81 (CH, C-aromatic), 121.21 (CH, C-aromatic), 125.73 (CH, C-aromatic), 127.06 (CH, C-aromatic), 128.20 (C, C-aromatic), 147.31 (C, C-aromatic), 147.39 (C, C-aromatic), 148.94 (C, C-aromatic), 149.14 (C, C-aromatic), 152.95 (C, C-aromatic), 165.12 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -62.82 ppm

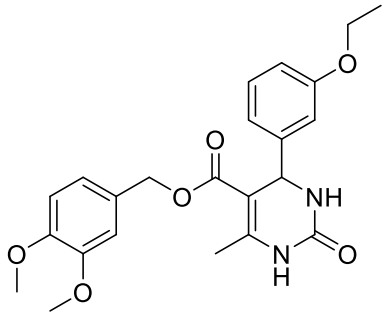
MS(ESI)$^+$: 451.2 $[M + H]^+$

m.p: (from ethanol/water) 140 - 142 °C

Synthesis of 3, 4-dimethoxybenzyl 4-(3-ethoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (205)

Chemical Formula: $C_{23}H_{26}N_2O_6$

Molecular Weight: 426.47



Procedure: 6

Yield: 38%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.38 – 1.43 (m, 3H), 2.37 (s, 3H), 3.81 (s, 3H), 3.89 (s, 3H), 3.92 – 3.97 (m, 2H), 4.98 (d, *J* = 12.10 Hz, 1H), 5.04 (d, *J* = 12.11 Hz, 1H), 5.37 (d, *J* = 2.69, 1H), 5.67 (s, 1H), 6.71 (m, 1H), 6.76 – 6.81 (m, 4H), 6.84 (m, 1H), 7.19 (td, *J* = 0.82, 7.64 Hz, 1H), 7.88 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.74 (CH_3, C-aliphatic), 55.89 (CH_3, C-aliphatic), 55.92 (CH_2, C-aliphatic), 56.04 (CH_3, C-aliphatic), 56.15 (CH_3, C-aliphatic), 60.76 (CH, C-aliphatic), 66.14 (CH_2, C-aliphatic), 100.87 (C, C-aromatic), 103.66 (CH, C-aromatic), 111.03 (CH, C-aromatic), 111.68 (CH, C-aromatic), 121.00 (CH, C-aromatic), 128.43 (C, C-aromatic), 137.78 (C, C-aromatic), 139.19 (C, C-aromatic), 146.90 (C, C-aromatic), 148.95 (C, C-aromatic), 149.04 (C, C-aromatic), 153.15 (C, C-aromatic), 153.39 (C, C-aromatic), 165.50 (C, C-aromatic) ppm.

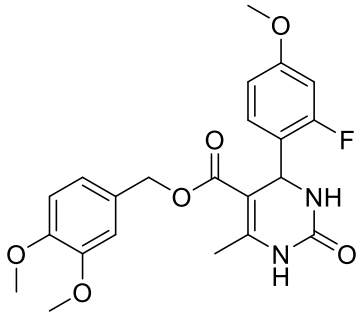
MS (ESI)$^+$: 427.2 $[M + H]^+$

m.p: (from ethanol/water) 144 – 146 °C

Synthesis of 3, 4-dimethoxybenzyl 4-(2-fluoro-4-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (206)

Chemical Formula: $C_{22}H_{23}FN_2O_6$

Molecular Weight: 430.43



Procedure: 6

Yield: 25 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.42 (s, 3H), 3.79 (s, 3H), 3.80 (s, 3H), 3.88 (s, 3H), 4.96 (d, J = 12.1 Hz, 1H), 5.04 (d, J = 12.1 Hz, 1H), 5.48 (s, 1H), 5.69 (d, J = 2.5 Hz, 1H), 6.63 – 6.51 (m, 2H), 6.65 (t, J = 10.1 Hz, 1H), 6.75 (dt, J = 5.0, 8.2 Hz, 2H), 7.06 (dd, J = 5.5, 12.2 Hz, 1H), 7.81 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.71 (CH_3, C-aliphatic), 49.34 (CH_3, C-aliphatic), 49.92 (CH, C-aliphatic), 55.77 (CH_3, C-aliphatic), 55.89 (CH_3, C-aliphatic), 65.87 (CH_2, C-aliphatic), 98.67 (C, C-aromatic), 109.94 (CH, C-aromatic), 110.88 (CH, C-aromatic), 111.45 (CH, C-aromatic), 120.87 (CH, C-aromatic), 121.76 (CH, C-aromatic), 121.87 (CH, C-aromatic), 128.57 (C, C-aromatic), 128.71 (C, C-aromatic), 148.89 (CH, C-aromatic), 148.92 (C, C-aromatic), 152.65 (C, C-aromatic), 159.91 (C, C-aromatic), 160.61 (C, C-aromatic), 160.70 (C, C-aromatic), 161.87 (C, C-aromatic), 165.17 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -117.69 ppm.

MS(ESI)$^{+}$: 431.2 [M + H^{+}]

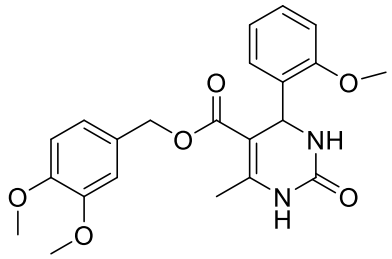
m.p: (from hot ethanol) 160 – 162 °C

HPLC (method 1): retention time 14.45 minutes

Synthesis of 3, 4-dimethoxybenzyl 4-(2-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (207)

Chemical Formula: $C_{22}H_{24}N_2O_6$

Molecular Weight: 412.44



Procedure: 6

Yield: 73%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.36 – 2.38 (s, 3H), 3.74 (s, 3H), 3.80 (s, 3H), 3.90 (s, 3H), 4.98 (d, *J* = 12.09 Hz, 1H), 5.05 (d, *J* = 12.10 Hz, 1H), 5.38 (d, *J* = 2.74 Hz, 1H), 5.67 (d, *J* = 0.34, 1H), 6.71 (d, *J* = 1.75, 1H), 6.79 (m, 4H), 6.86 (m, 1H), 7.19 – 7.23 (m, 1H), 7.78 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.81 (CH_3, C-aliphatic), 55.14 (CH_3, C-aliphatic), 55.63 (CH, C-aliphatic), 55.83 (CH_3, C-aliphatic), 55.91 (CH_3, C-aliphatic), 66.08 (CH_2, C-aliphatic), 100.91 (C, C-aromatic), 110.97 (CH, C-aromatic), 111.64 (CH, C-aromatic), 112.62 (CH, C-aromatic), 112.98 (CH, C-aromatic), 118.81 (CH, C-aromatic), 121.07 (CH, C-aromatic), 128.49 (C, C-aromatic), 129.82 (CH, C-aromatic), 145.06 (C, C-aromatic), 146.90 (C, C-aromatic), 148.94 (C, C-aromatic), 148.98 (C, C-aromatic), 152.89 (C, C-aromatic), 159.85 (C, C-aromatic), 165.42 (C, C-aromatic) ppm.

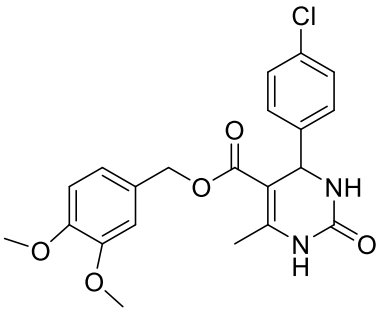
MS(ESI)$^+$: 413.2 $[M + H]^+$

m.p: (from ethanol/water) 150 - 152 °C

Synthesis of 3, 4-dimethoxybenzyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (208)

Chemical Formula: $C_{21}H_{21}ClN_2O_5$

Molecular Weight: 416.86



Procedure: 6

Yield: 34 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.38 (s, 3H), 3.83 (s, 3H), 3.91 (s, 3H), 4.96 (d, J = 12.0 Hz, 1H), 5.06 (d, J = 12.0 Hz, 1H), 5.39 (d, J = 2.2 Hz, 1H), 5.63 (s, 1H), 6.71 (s, 1H), 6.75 (d, J = 8.2 Hz, 1H), 6.82 (d, J = 8.1 Hz, 1H), 7.19 (d, J = 8.4 Hz, 2H), 7.25 (d, J = 8.4 Hz, 2H), 7.74 (s, 1H) ppm.

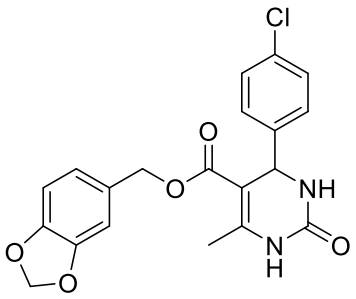
^{13}C-NMR ($CDCl_3$): δ 18.86 (CH_3, C-aliphatic), 55.21 (CH, C-aliphatic), 55.86 (CH_3, C-aliphatic), 55.93 (CH_3, C-aliphatic), 66.20 (CH_2, C-aliphatic), 100.85 (C, C-aromatic), 110.98 (CH, C-aromatic), 111.78 (CH, C-aromatic), 121.21 (CH, C-aromatic), 128.08 (CH, C-aromatic), 128.29 (CH, C-aromatic), 128.87 (C, C-aromatic), 133.80 (C, C-aromatic), 142.05 (C, C-aromatic), 146.88 (C, C-aromatic), 148.94 (C, C-aromatic), 149.12 (C, C-aromatic), 152.66 (C, C-aromatic), 165.19 (C, C-aromatic) ppm.

MS(ESI)$^+$: 417.1 $[M + H]^+$

m.p: (from hot ethanol) 158 – 160 °C

Synthesis of benzo[d][1,3]dioxol-5-ylmethyl 4-(4-chlorophenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (209)

Chemical Formula: $C_{20}H_{17}ClN_2O_5$
Molecular Weight: 400.82



Procedure: 6

Yield: 44 %

State: white powder

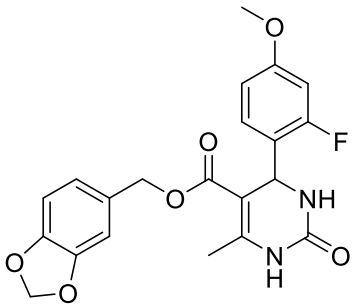
^{1}H-NMR ($CDCl_3$): δ 2.27 (s, 3H), 4.81 (d, J = 12.1 Hz, 1H), 4.81(d, J = 12.1 Hz, 1H), 4.93 (d, J = 2.6 Hz, 1H), 5.28 (d, J = 2.8 Hz, 1H), 5.72 (s, 1H), 5.85 – 5.93 (m, 2H), 6.51 (d, J = 1.4 Hz, 1H), 6.52 – 6.62 (m, 1H), 6.68 (d, J = 7.9 Hz, 1H), 7.09 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.5 Hz, 1H), 8.06 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.79 (CH_3, C-aliphatic), 53.1 (CH, C-aliphatic), 55.16 (CH_2, C-aliphatic), 65.98 (CH_2, C-aliphatic), 100.60 (C, C-aromatic), 108.12 (C, C-aromatic), 108.92 (CH, C-aromatic), 112.06 (CH, C-aromatic), 122.16 (CH, C-aromatic), 128.10 (CH, C-aromatic), 128.90 (CH, C-aromatic), 129.56 (C, C-aromatic), 133.81 (C, C-aromatic), 142.03 (C, C-aromatic), 147.56 (C, C-aromatic), 147.72 (C, C-aromatic), 152.98 (C, C-aromatic), 165.14 (C, C-aromatic) ppm.
MS(ESI)$^-$: 399.1 $[M - H]^-$
m.p: (from hot ethanol) 132 – 134 °C
HPLC (method 1): retention time 16.07 minutes

Synthesis of benzo[d][1,3]dioxol-5-ylmethyl 4-(2-fluoro-4-methoxyphenyl)-6-methyl-2-oxo-1, 2, 3, 4-tetrahydropyrimidine-5-carboxylate (210)

Chemical Formula: $C_{21}H_{19}FN_2O_6$

Molecular Weight: 414.39



Procedure: 6

Yield: 35 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.43 (s, 3H), 3.81 (s, 3H), 4.90 – 4.98 (m, 2H), 5.36 (s, 1H), 5.69 (d, J = 2.4 Hz, 1H), 5.96 (dd, J = 1.4, 3.1 Hz, 2H), 6.54 (d, J = 1.5 Hz, 1H), 6.59 – 6.68 (m, 3H), 6.71 (d, J = 7.9 Hz, 1H), 7.07 (dd, J = 6.5, 11.2 Hz, 1H), 7.17 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 18.85 (CH_3, C-aliphatic), 49.40 (CH_3, C-aliphatic), 55.57 (CH, C-aliphatic), 65.73 (CH_2, C-aliphatic), 98.78 (C, C-aromatic), 101.74 (CH, C-aromatic), 101.95 (CH, C-aromatic), 108.00 (CH, C-aromatic), 108.67 (CH, C-aromatic), 110.05 (C, C-aromatic), 121.83 (CH, C-aromatic), 128.70 (CH, C-aromatic), 129.79 (C, C-aromatic), 147.74 (C, C-aromatic), 152.11 (C, C-aromatic), 159.97 (C, C-aromatic), 160.70 (C, C-aromatic), 160.91 (C, C-aromatic), 161.91 (C, C-aromatic), 165.02 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -117.82 ppm.

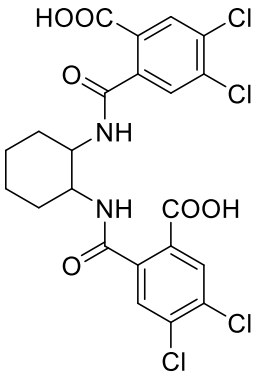
MS(ESI)$^-$: 415.1 $[M - H]^-$

m.p: (from hot ethanol) 138 - 140°C

HPLC (method 1): retention time 16.2 minutes

Synthesis of 6, 6'-((cyclohexane-1,2-diylbis(azanediyl))bis(carbonyl))bis(3,4-dichlorobenzoic acid) (211)

Chemical Formula: $C_{22}H_{18}Cl_4N_2O_6$
Molecular Weight: 548.19



Procedure: 7

Yield: 35 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.18 (t, J = 7.1 Hz, 2H), 1.67 (d, J = 6.1 Hz, 2H), 4.04 (q, J = 7.1 Hz, 1H), 7.90 – 8.10 (m, 2H), 8.22 (d, J = 8.6 Hz, 2H), 13.41 (s, 1H, OH) ppm.

^{13}C-NMR ($CDCl_3$): δ 21.70 (CH_2, C-aliphatic). 27.46 (CH_2, C-aliphatic), 48.81 (CH, C-aliphatic), 129.98 (CH, C-aromatic), 130.24 (C, C-aromatic), 130.54 (CH, C-aromatic), 133.50 (C, C-aromatic), 138.96 (C, C-aromatic), 166.00 (C, C-aromatic), 170.28 (C, C-aromatic) ppm.

MS(ESI)$^+$: 549.0 $[M + H]^+$

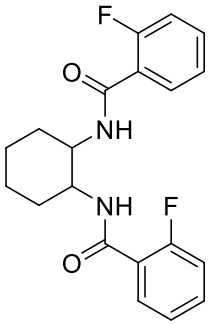
m.p: (from hot ethanol) 130 - 132°C

HPLC (method 1): retention time 15.7 minutes

Synthesis of (R, R)-*N, N'*-(cyclohexane-1, 2-diyl)bis(2-fluorobenzamide) (212)

Chemical Formula: $C_{20}H_{20}F_2N_2O_2$

Molecular Weight: 358.39



Procedure: 8

Yield: 39 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.29 (s, 1H), 1.45 (s, 1H), 1.86 (s, 1H), 2.27 (s, 1H), 4.09 (s, 1H, -CH), 6.94-7.01 (s, 1H, NH), 7.02 – 7.15 (m, 1H), 7.20 (dd, J = 4.3, 9.6 Hz, 1H), 7.44 (d, J = 5.4 Hz, 1H), 7.93 (t, J = 7.1 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.78 (CH_2, C-aliphatic), 32.50 (CH_2, C-aliphatic), 53.83 (CH, C-aliphatic), 116.00 (CH, C-aromatic, d = 25 Hz), 121.36 (C, C-aromatic, d, J = 11.25 Hz), 124.52 (CH, C-aromatic), 131.57 (CH, C-aromatic), 133.02 (CH, C-aromatic, d = 10 Hz), 159.46 (C, C-aromatic), 161.43 (C, C-aromatic), 163.86 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -113.15 ppm.

MS(ESI)$^+$: 381.2 $[M + Na]^+$

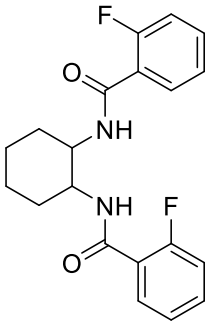
m.p: 187-189 °C

HPLC (method 1): retention time 15.59 minutes

Synthesis of (S, S)-*N, N'*-(cyclohexane-1, 2-diyl)bis(2-fluorobenzamide) (213)

Chemical Formula: $C_{20}H_{20}F_2N_2O_2$

Molecular Weight: 358.39



Procedure: 8

Yield: 58 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.21 (s, 1H), 1.36 (s, 1H), 1.76 (s, 1H), 2.17 (s, 1H), 4.01 (s, 1H, -CH), 6.89-7.04 (s, 1H, NH), 7.04 – 7.13 (m, 1H), 7.33 (dd, J = 6.1, 13.3 Hz, 1H), 7.39 (d, J = 5.4 Hz, 1H), 7.83 (t, J = 7.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.78 (CH_2, C-aliphatic), 32.51 (CH_2, C-aliphatic), 53.97 (CH, C-aliphatic), 115.99 (CH, C-aromatic, d, J = 23.75 Hz), 121.51 (C, C-aromatic, d, J = 11.25 Hz), 124.50 (CH, C-aromatic), 131.58 (CH, C-aromatic), 132.68 (CH, C-aromatic), 159.45 (C, C-aromatic), 161.43 (C, C-aromatic), 164.01 (C, C-aromatic) ppm.

^{19}F-NMR (CDCl3): δ -113.10 ppm.

MS(ESI)$^{+}$: 381.1 $[M + Na]^{+}$

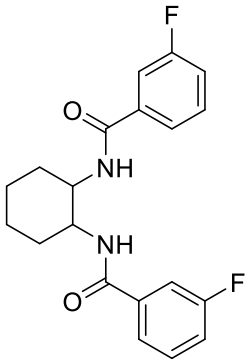
m.p: 180-182 °C

HPLC (method 1): retention time 15.57 minutes

Synthesis of (R, R)-*N, N′* − (cyclohexane-1, 2-diyl)bis(3-fluorobenzamide) (214)

Chemical Formula: $C_{20}H_{20}F_2N_2O_2$

Molecular Weight: 358.39



Procedure: 8

Yield: 51 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.47 (d, J = 7.2 Hz, 1H), 1.65 (s, 1H), 1.90 (s, 1H), 2.25 (d, J = 7.0 Hz, 1H), 4.04 (s, 1H, CH), 6.88 (s, 1H, NH), 7.15 (td, J = 1.7, 8.3 Hz, 1H), 7.27 – 7.38 (m, 1H), 7.45 – 7.55 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.81 (CH_2, C-aliphatic), 32.27 (CH_2, C-aliphatic), 54.61 (CH, C-aliphatic), 114.45 (CH, C-aromatic, d, J = 23.75 Hz), 118.50 (CH, C-aromatic, d, J = 21.25 Hz), 122.32 (CH, C-aromatic), 130.15 (CH, C-aromatic), 136.45 (C, C-aromatic, d, J = 6.25 Hz), 161.74 (C, C-aromatic), 163.71 (C, C-aromatic), 166.94 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.80 ppm.

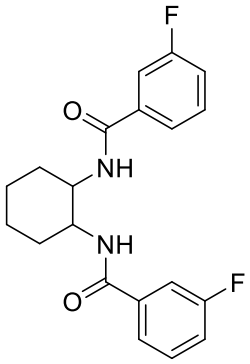
MS(ESI)$^+$: 359.2 $[M + H]^+$

m.p: < 200°C

Synthesis of (S,S)-*N,N′*−(cyclohexane-1,2-diyl)bis(3-fluorobenzamide) (215)

Chemical Formula: $C_{20}H_{20}F_2N_2O_2$

Molecular Weight: 358.39



Procedure: 8

Yield: 31 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.37-1.55 (bs, 2H), 1.87 (t, *J* = 18.4 Hz, 1H), 2.22 (t, J = 7.0 Hz, 1H), 4.05 (s, 1H, CH), 6.98 (s, 1H, NH), 7.13 (dd, J = 11.3, 13.0 Hz, 1H), 7.23 – 7.37 (m, 1H), 7.41 – 7.50 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.79 (CH_2, C-aliphatic), 32.30 (CH_2, C-aliphatic), 54.58 (CH, C-aliphatic), 114.32 (CH, C-aromatic, d, J = 25.1 Hz), 118.41 (CH, C-aromatic, d, J = 21.25 Hz), 122.28 (CH, C-aromatic), 130.18 (CH, C-aromatic), 136.40 (C, C-aromatic), 161.72 (C, C-aromatic), 163.69 (C, C-aromatic), 167.99 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl^3$): δ -111.79 ppm.

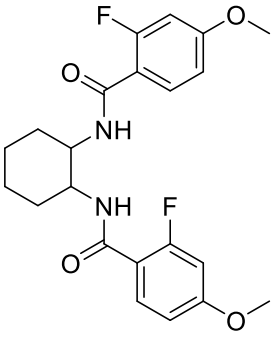
MS(ESI)$^+$: 381.1 $[M + Na]^+$

m.p: < 200°C

Synthesis of (R, R)-*N, N'*-(cyclohexane-1,2-diyl)bis(2-fluoro-4-methoxybenzamide) (216)

Chemical Formula: $C_{22}H_{24}F_2N_2O_4$

Molecular Weight: 418.44



Procedure: 8

Yield: 19 %

State: white powder

^{1}H-NMR (Pyridine-d$_5$): δ 2.35 (s, 1H), 2.66 (s, 2H), 3.16 (bs, 3H), 4.70 (t, J = 18.8 Hz, 2H), 5.44 (s, 1H), 7.50 – 7.59 (m, 1s), 7.57 – 7.66 (m, 1H, NH), 9.01 (s, 1H) ppm.

^{13}C-NMR (Pyridine-d$_5$): δ 26.82 (CH_2, C-aliphatic), 34.42 (CH_2, C-aliphatic), 55.82 (CH, C-aliphatic), 57.29 (CH_3, C-aliphatic), 103.51 (CH, C-aromatic, d, J = 27.5 Hz), 112.41 (CH, C-aromatic), 117.86 (C, C-aromatic, d, J = 13.75 Hz), 134.07 (CH, C-aromatic), 162.37 (C, C-aromatic), 164.35 (C, C-aromatic), 164.86 (C, C-aromatic, d, J = 13.75 Hz), 166.13 (C, C-aromatic) ppm.

^{19}F-NMR (Pyridine-d$_5$): δ -110.51 ppm.

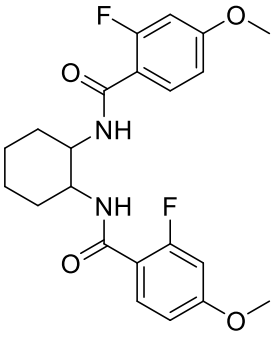
MS(ESI)$^+$: 419.2 $[M + H]^+$

m.p: < 200 °C

Synthesis of (S, S)-*N, N'*-(cyclohexane-1, 2-diyl) bis (2-fluoro-4-methoxybenzamide) (217)

Chemical Formula: $C_{22}H_{24}F_2N_2O_4$

Molecular Weight: 418.44



Procedure: 8

Yield: 33 %

State: white powder

^{1}H-NMR (Pyridine-d_5): δ 1.22 (t, J = 9.9 Hz, 2H), 1.50 (d, J = 10.1 Hz, 2H), 2.25 (d, J = 13.1 Hz, 1H), 3.56 (s, 3H), 6.63 – 6.88 (m, 1H), 8.13 (dt, J = 8.0, 14.4 Hz, 1H), 8.53 (s, 1H) ppm.

^{13}C-NMR (Pyridine-d_5): δ 26.82 (CH_2, C-aliphatic), 34.42 (CH_2, C-aliphatic), 55.81 (CH, C-aliphatic), 57.29 (CH_3, C-aliphatic), 103.51 (CH, C-aromatic, d, J = 27.5 Hz), 112.39 (CH, C-aromatic), 117.90 (C, C-aromatic, d, J = 13.75 Hz), 134.09 (CH, C-aromatic), 162.37 (C, C-aromatic), 164.35 (C, C-aromatic), 164.86 (C, C-aromatic, d, J = 12.5 Hz), 166.16 (C, C-aromatic) ppm.

^{19}F-NMR (Pyridine-d_5): δ -109.34 ppm.

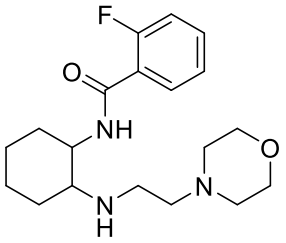
MS(ESI)$^{+}$: 419.2 [M + H^{+}]

m.p: < 190 °C

Synthesis of 2-fluoro-N-(2-((2-morpholinoethyl)amino)cyclohexyl)benzamide (218)

Chemical Formula: $C_{19}H_{28}FN_3O_2$

Molecular Weight: 349.45



Procedure: A stirred solution of 4-(2-chloro ethyl)morpholine (1 equivalent), compound **307** (1 equivalent) in *N*, *N*-dimethylformamide (4 ml/ mmol), potassium iodide (0.2 equivalent) and potassium carbonate (4 equivalents) was heated at 80 °C for 16 hours. The reaction mixture was diluted with water, extracted with ethyl acetate, dried over $MgSO_4$, filtered and evaporated to give a yellow oil purified by silica gel column chromatography.

Yield: 31 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 1.14 – 1.38 (m, 4H), 1.58 (s, 1H), 1.72 (d, J = 7.2 Hz, 2H), 2.07 (dd, J = 12.8, 25.2 Hz, 2H), 2.32 (d, J = 4.5 Hz, 3H), 2.42 (t, J = 5.8 Hz, 2H), 2.82 (d, J = 0.5 Hz, 1H), 2.89 (s, 1H), 3.41 – 3.49 (m, 1H), 3.56 (t, J = 4.6 Hz, 3H), 3.85 (d, J = 8.7 Hz, 1H), 4.03 (dd, J = 5.7, 11.8 Hz, 2H), 5.07 (d, J = 8.3 Hz, 1H), 6.75 – 6.84 (m, 1H), 7.05 (dd, J = 8.7, 11.4 Hz, 1H), 7.30-7.42 (m, 1H), 7.95 (dd, J = 1.8, 7.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.72 (CH_2, C-aliphatic), 24.80 (CH_2, C-aliphatic), 32.45 (CH_2, C-aliphatic), 32.86 (CH_2, C-aliphatic), 53.71 (CH_2, C-aliphatic), 54.17 (CH, C-aliphatic), 55.26 (CH, C-aliphatic), 57.36 (CH_2, C-aliphatic), 61.85 (CH_2, C-aliphatic), 66.83 (CH_2, C-aliphatic), 116.06 (CH, C-aromatic, d, J = 23.75 Hz), 121.25 (C, C-aromatic, d, J = 11.25 Hz), 124.68 (CH, C-aromatic), 131.77 (CH, C-aromatic), 133.28 (CH, C-aromatic, d, J = 10 Hz), 156.70 (C, C-aromatic), 159.53 (C, C-aromatic), 163.85 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -113.03 ppm.

MS(ESI)$^+$: 350.2 [M + H^+]

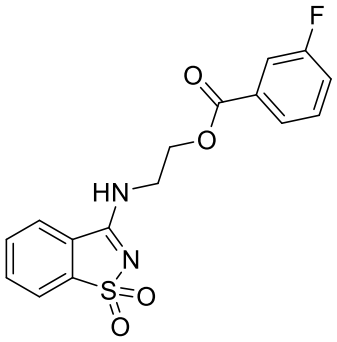
m.p: 84 – 86 °C

HPLC (method 1): retention time 10.67 minutes

Synthesis of 2-((1, 1-dioxidobenzo[d]wasothiazol-3-yl)amino)ethyl 3-fluorobenzoate (219)

Chemical Formula: $C_{16}H_{13}N_2O_4S$

Molecular Weight: 348.06



Procedure: 11

Yield: 14 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 3.91 (dd, J = 5.5, 10.9 Hz, 2H), 4.74 (t, J = 5.3 Hz, 2H), 7.020 – 7.15 (m, 1H), 7.28-7.32 (m, 1H), 7.44-7.51 (m, 2H), 7.64 – 7.72 (m, 2H), 7.85 – 7.76 (m, 2H), 10.89 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 40.77 (CH_2, C-aliphatic), 57.63 (CH_2, C-aliphatic), 114.85 (CH, C-aromatic), 116.65 (CH, C-aromatic), 118.12 (CH, C-aromatic), 118.42 (CH, C-aromatic), 124.27 (CH, C-aromatic), 126.76 (CH, C-aromatic), 128.35 (CH, C-aromatic), 129.06 (CH, C-aromatic), 130.56 (C, C-aromatic), 158.53 (C, C-aromatic), 161.06 (C, C-aromatic), 162.20 (C, C-aromatic), 164.20 (C, C-aromatic), 165.90 (C, C-aromatic) ppm.

MS(ESI)$^+$: 349.1 $[M + H]^+$

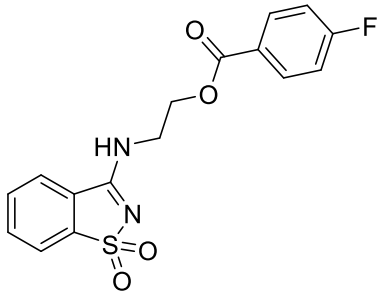
m.p: 110 - 112 °C

^{19}F-NMR: δ -108.06 ppm.

Synthesis of 2-((1, 1-dioxidobenzo[d]wasothiazol-3-yl)amino)ethyl 4-fluorobenzoate (220)

Chemical Formula: $C_{16}H_{13}N_2O_4S$

Molecular Weight: 348.06



Procedure: 11

Yield: 25 %

State: pale yellow powder

^{1}H-NMR ($CDCl_3$): δ 3.14 (t, J = 6.5 Hz, 2H), 3.96 – 4.06 (m, 2H), 7.06 – 7.14 (m, 1H), 7.28 – 7.36 (m, 1H), 7.44 – 7.54 (m, 2H), 7.60 – 7.65 (m, 1H), 7.69 (dd, J = 7.5, 11.0 Hz, 2H), 7.79 (d, J = 7.6 Hz, 1H), 11.01 ppm.

^{13}C-NMR ($CDCl_3$): δ 45.84 (CH_2, C-aliphatic), 58.65 (CH_2, C-aliphatic), 120.14 (CH, C-aromatic), 122.85 (CH, C-aromatic), 123.35 (CH, C-aromatic), 125.67 (CH, C-aromatic), 131.96 (CH, C-aromatic), 132.22 (CH, C-aromatic), 132.52 (CH, C-aromatic), 132.72 (CH, C-aromatic), 133.34 (C, C-aromatic), 158.91 (C, C-aromatic), 159.82 (C, C-aromatic), 161.78 (C, C-aromatic), 165.20 (C, C-aromatic), 166.90 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.85 ppm.

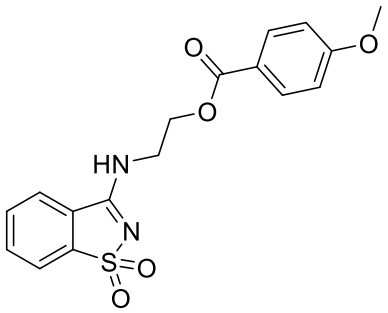
MS(ESI)$^+$: 349 $[M + H]^+$

m.p: 101 - 103 °C

Synthesis of 2-((1, 1-dioxidobenzo[d]wasothiazol-3-yl)amino)ethyl 4-methoxybenzoate (221)

Chemical Formula: $C_{17}H_{16}NO_5S$

Molecular Weight: 360.38



Procedure: 11

Yield: 16 %

State: colourless oil

^{1}H-NMR ($CDCl_3$): δ 3.04 (d, J = 6.1 Hz, 2H). 3.31 (s, 3H), 3.36 (s, 2H), 7.41 (s, 2H), 7.60 (t, J = 21.5 Hz, 4H), 7.70-7.79 (m, 1H), 8.01 (d, J = 6.1 Hz, 1H), 8.24 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 42.51 (CH_2, C-aliphatic), 55.29 (CH_3, C-aliphatic), 64.32 (CH_2, C-aliphatic), 114.61 (CH, C-aromatic), 127.25 (CH, C-aromatic), 128.34 (CH, C-aromatic), 131.80 (CH, C-aromatic), 132.73 (CH, C-aromatic), 133.22 (CH, C-aromatic), 133.86 (C, C-aromatic), 135.22 (C, C-aromatic), 157.74 (C, C-aromatic), 158.56 (C, C-aromatic), 163.98 (C, C-aromatic), 165.31 (C, C-aromatic) ppm.

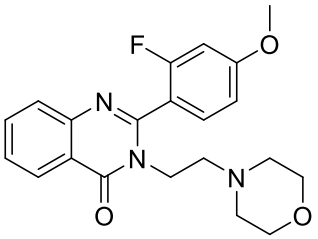
m.p: - °C

MS(ESI)$^{+}$: 361[M + H^{+}]

Synthesis of 2-(2-fluoro-4-methoxyphenyl)-3-(2-morpholinoethyl)quinazolin-4(3H)-one (222)

Chemical Formula: $C_{21}H_{22}FN_3O_3$

Molecular Weight: 383.42



Procedure: 12

Yield: 62%

State: colourless oil

^{1}H-NMR ($CDCl_3$): δ 2.18 (s, 4H), 2.36 – 2.50 (m, 2H), 3.42 – 3.52 (m, 4H), 3.77 (s, 3H), 3.81 – 3.89 (m, 1H), 4.27 (s, 1H), 6.61 – 6.70 (m, 1H), 6.76 – 6.81 (m, 1H), 7.33 – 7.48 (m, 2H), 7.61 – 7.72 (m, 2H), 8.23 (d, J = 8.0 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 35.80 (CH_2, C-aliphatic), 53,58 (CH_2, C-aliphatic), 55.71 (CH_3, C-aliphatic), 56.62 (CH_2, C-aliphatic), 66.80 (CH_2, C-aliphatic), 117.03 (CH, C-aromatic), 117.37 (C, C-aromatic, d, J = 20 Hz), 119.07 (C, C-aromatic, d, J = 8.75 Hz), 124.32 (CH, C-aromatic), 127.46 (CH, C-aromatic), 128.59 (CH, C-aromatic), 128.78 (CH, C-aromatic), 131.16 (CH, C-aromatic), 133.98 (CH, C-aromatic, d, J = 8.75 Hz), 136.63 (C, C-aromatic), 146.71 (C, C-aromatic), 154.86 (C, C-aromatic), 160.38 (C, C-aromatic), 162.45 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 109.215 ppm

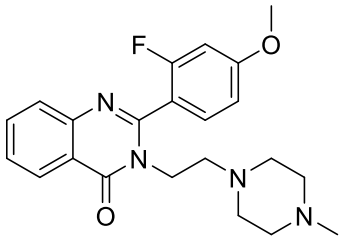
MS (ESI)$^-$: 382.2 $[M - H]^-$

m.p.:-

HPLC (method 1): retention time 10.45 minutes

Synthesis of 2-(2-fluoro-4-methoxyphenyl)-3-(2-(4-methylpiperazin-1-yl)ethyl)quinazolin-4(3H)-one (223)
Chemical Formula: $C_{22}H_{25}FN_4O_2$

Molecular Weight: 396.47



Procedure: 12

Yield: 52%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.20 (s, 3H), 2.30 (bs, 8H), 2.47 (t, J = 7.1 Hz, 2H), 3.80 (s, 3H), 4.28 (s, 2H), 6.67 (dd, J = 2.4, 11.5 Hz, 1H), 6.78 (dd, J = 2.4, 8.5 Hz, 1H), 7.38 (t, J = 8.4 Hz, 1H), 7.45 (ddd, J = 1.4, 6.9, 8.2 Hz, 1H), 7.60 – 7.73 (m, 2H), 8.18 – 8.31 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 42.72 (CH_2, C-aliphatic), 45.73 (CH_3, C-aliphatic), 52.75 (CH_2, C-aliphatic), 54.85 (CH_2, C-aliphatic), 55.88 (CH_3, C-aliphatic), 55.90 (CH_2, C-aliphatic), 102.00 (CH, C-aromatic, d, J = 25 Hz), 110.80 (CH, C-aromatic), 120.97 (C, C-aromatic), 126.80 (CH, C-aromatic), 127.25 (CH, C-aromatic), 127.53 (CH, C-aromatic), 131.13 (CH, C-aromatic), 134.36 (CH, C-aromatic), 147.25 (C, C-aromatic), 151.63 (C, C-aromatic), 158.95 (C, C-aromatic), 160.92 (C, C-aromatic), 162.06 (C, C-aromatic), 162.59 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 111.80 ppm

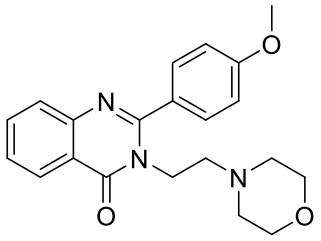
MS (ESI)$^-$: 395.2 $[M - H]^-$

m.p.: 106 – 108 °C

Synthesis of 2-(4-methoxyphenyl)-3-(2-morpholinoethyl)quinazolin-4(3H)-one (224)

Chemical Formula: $C_{21}H_{22}N_3O_3$

Molecular Weight: 365.43



Procedure: 12

Yield: 36 %

State: orange powder

^{1}H-NMR ($CDCl_3$): δ 2.32 – 2.49 (m, 2H), 2.53 (dd, J = 12.7, 18.7 Hz, 2H), 3.47 (dd, J = 4.4, 8.9 Hz, 4H), 3.56 – 3.72 (m, 2H), 3.77 (s, 3H), 4.13 (t, J = 6.9 Hz, 2H), 6.77 – 6.96 (m, 1H), 7.39 – 7.52 (m, 1H), 7.59 – 7.74 (m, 1H), 8.22 (d, J = 8.0 Hz, 1H), 8.72 (dd, J = 8.3, 18.1 Hz, 1H), 12.05 (s, 1H) ppm.

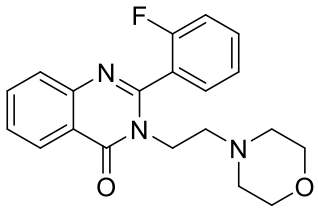
^{13}C-NMR ($CDCl_3$): δ 53.78 (CH_2, C-aliphatic), 53.95 (CH_2, C-aliphatic), 55.42 (CH_3, C-aliphatic), 55.60 (CH_2, C-aliphatic), 66.87 (CH_2, C-aliphatic), 114.37 (CH, C-aromatic), 119.07 (C, C-aromatic), 127.87 (CH, C-aromatic), 128.79 (CH, C-aromatic), 129.72 (CH, C-aromatic), 130.90 (CH, C-aromatic), 132.62 (CH, C-aromatic), 147.22 (C, C-aromatic), 156.09 (C, C-aromatic), 160.73 (C, C-aromatic), 162.55 (C, C-aromatic), 165.14 (C, C-aromatic) ppm.

MS (ESI)$^-$: 364.2 $[M - H]^-$

m.p.: 70 - 72 °C

Synthesis of 2-(2-fluoro)-3-(2-morpholinoethyl)quinazolin-4(3H)-one (225)
Chemical Formula: $C_{20}H_{20}FN_3O_2$

Molecular Weight: 353.40



Procedure: 12

Yield: 63 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 2.16 (dd, J = 4.6, 9.6 Hz, 4H), 2.40 – 2.55 (m, 2H), 3.48 (t, J = 4.6 Hz, 4H), 3.81 (dt, J = 7.1, 14.0 Hz, 1H), 4.19 – 4.32 (m, 1H), 7.08 – 7.18 (m, 1H), 7.23 – 7.32 (m, 1H), 7.39 – 7.57 (m, 3H), 7.63 – 7.78 (m, 2H), 8.22 – 8.33 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 42.59 (CH_2, C-aliphatic), 53.59 (CH_2, C-aliphatic), 56.52 (CH_2, C-aliphatic), 66.83 (CH_2, C-aliphatic), 116.22 (CH, C-aromatic, d, J = 20 Hz), 123.51 (C, C-aromatic), 123.64 (CH, C-aromatic), 124.88 (CH, C-aromatic), 126.83 (CH, C-aromatic), 127.50 (CH, C-aromatic), 130.66 (CH, C-aromatic), 132.18 (CH, C-aromatic), 134.51 (CH, C-aromatic), 147.18 (C, C-aromatic), 151.53 (C, C-aromatic), 158.05 (C, C-aromatic), 160.03 (C, C-aromatic), 161.94 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 113.26 ppm.

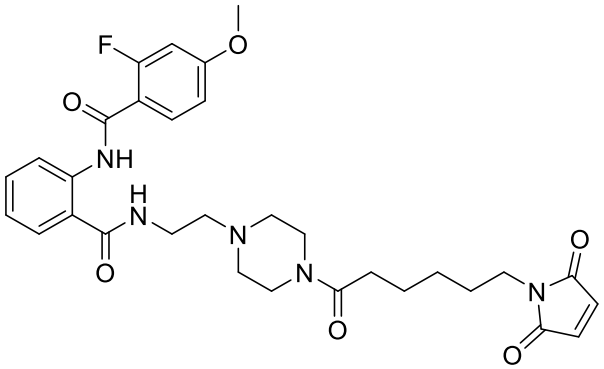
MS(ESI)$^+$: 354.2 $[M + H]^+$

m.p.: 90 – 92 °C

Synthesis of N-(2-((2-(4-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl)piperazin-1-yl)ethyl)carbamoyl)phenyl)-2-fluoro-4-methoxybenzamide (227)

Chemical Formula: $C_{31}H_{36}FN_5O_6$

Molecular Weight: 593.66



Procedure: A solution was prepared by adding sequentially 6 maleimido hexanoic acid (1 equivalent), (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) (TBTU, 1.2 equivalents) *N*,*N*-diisopropylethylamine (1.2 equivalent) in DMF (1 mL/mmol). After 20 minutes, compound **99** (1 equivalent) was added and the reaction mixture was stirred at 25 °C for 16 hours. The reaction mixture was diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude compound (as a brown oil) was send to Nanotethers for the final purification.

^{1}H-NMR ($CDCl_3$): δ 1.48 – 1.64 (m, 6H), 1.76 – 1.83 (m, 2H), 2.14 – 2.24 (m, 2H), 2.36 – 2.47 (m, 2H), 2.56 (t, J = 6.0 Hz, 2H), 2.69 – 2.74 (m, 4H), 3.03 (t, J = 6.7 Hz, 1H), 3.34 – 3.56 (m, 5H), 3.78 (s, 3H), 6.58 – 6.63 (m, 1H), 6.71 – 6.76 (m, 1H), 6.98 – 7.01 (m, 1H), 7.32 – 7.53 (m, 1H), 7.64 – 7.75 (m, 1H), 7.86 – 7.97 (m, 1H), 8.23 – 8.37 (m, 1H), 8.60 (dd, J = 6.3, 14.0 Hz, 1H), 11.55 (d, J = 8.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 25.92 (CH_2, C-aliphatic), 28.29 (CH_2, C-aliphatic), 34.78 (CH_2, C-aliphatic), 36.29 (CH_2, C-aliphatic), 37.30 (CH_2, C-aliphatic), 38.54 (CH_2, C-aliphatic), 41.85 (CH_2, C-aliphatic), 45.37 (CH_2, C-aliphatic), 52.98 (CH_2, C-aliphatic), 55.84 (CH_2, C-aliphatic), 56.31 (CH_2, C-aliphatic), 110.66 (CH, C-aromatic), 114.83 (CH, C-aromatic), 116.03 (CH, C-aromatic), 120.40 (CH, C-aromatic), 122.42 (CH, C-aromatic), 123.07 (C, C-aromatic), 128.74 (CH, C-aromatic), 133.02 (C, C-aromatic), 143.38 (C, C-aromatic), 160.51 (C, C-aromatic), 161.97 (C, C-aromatic), 162.50 (C, C-aromatic), 163.66 (C, C-aromatic), 163.75 (C, C-aromatic), 168.74 (C, C-aromatic), 169.27 (C, C-aromatic), 169.66 (C, C-aromatic) ppm.

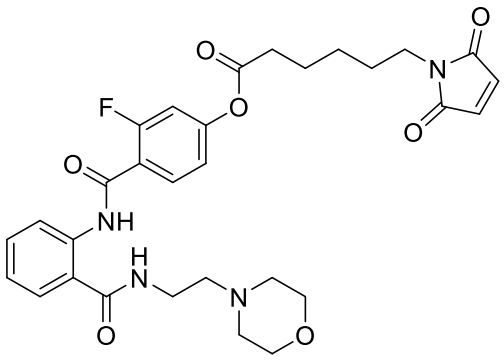
^{19}F-NMR ($CDCl_3$): δ -109.20 ppm.

MS(ESI)$^-$: 592.54 $[M - H]^-$

Synthesis of 3-fluoro-4-((2-((2-morpholinoethyl)carbamoyl)phenyl)carbamoyl)phenyl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (228)

Chemical Formula: $C_{30}H_{33}FN_4O_7$

Molecular Weight: 580.61



Procedure: A solution was repared by adding sequentially 6 maleimido hexanoic acid (1 equivalent), (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) (TBTU, 1.2 equivalents) *N*,*N*-diisopropylethylamine (1.2 equivalent) in DMF (1 mL/mmol). After 30 minutes, compound **127** (1 equivalent) was added and the reaction mixture was stirred at 25 °C for 18 hours. The reaction mixture was diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude compound (as a brown oil) was send to Nanotethers for the final purification.

^{1}H-NMR ($CDCl_3$): δ 1.48 – 1.70 (m, 4H), 2.23 (t, J = 7.5 Hz, 2H), 2.50 (d, J = 14.8 Hz, 4H), 2.60 (t, J = 5.6 Hz, 2H), 3.44 (d, J = 7.3 Hz, 3H), 3.59 (s, 2H), 3.65 – 3.73 (m, 3H), 5.23 (s, 4H), 6.56 – 6.70 (m, 4H), 7.09 (ddd, J = 4.1, 8.6, 10.1 Hz, 2H), 7.46 (dd, J = 4.4, 12.7 Hz, 2H), 7.83 (t, J = 8.8 Hz, 1H), 8.59 (t, J = 14.8 Hz, 1H), 11.30 (d, J = 8.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 24.40 (CH_2, C-aliphatic), 26.24 (CH_2, C-aliphatic), 28.22 (CH_2, C-aliphatic), 33.86 (CH_2, C-aliphatic), 36.03 (CH_2, C-aliphatic), 37.57 (CH_2, C-aliphatic), 51.55 (CH_2, C-aliphatic), 53.28 (CH_2, C-aliphatic), 56.53 (CH_2, C-aliphatic), 66.69 (CH_2, C-aliphatic), 103.41 (CH, C-aliphatic), 103.62 (CH, C-aliphatic), 122.15 (C, C-aromatic), 122.84 (CH, C-aromatic), 123.47 (CH, C-aromatic), 126.81 (CH, C-aromatic), 132.51 (CH, C-aromatic), 132.87 (CH, C-aromatic), 132.90 (CH, C-aromatic), 134.08 (CH, C-aromatic), 134.11 (C, C-aromatic), 138.86 (C, C-aromatic), 160.46 (C, C-aromatic), 161.53 (C, C-aromatic), 162.45 (C, C-aromatic), 169.37 (C, C-aromatic), 170.86 (C, C-aromatic) ppm.

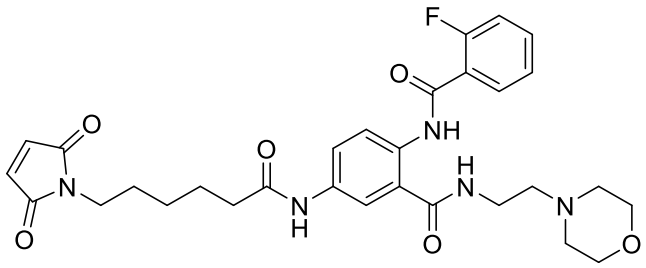
^{19}F-NMR ($CDCl_3$): δ -109.13 ppm.

MS(ESI)$^+$: 581.24 $[M + H]^+$

Synthesis of 5-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)-2-(2-fluorobenzamido)-N-(2-morpholinoethyl)benzamide (229)

Chemical Formula: $C_{30}H_{34}FN_5O_6$

Molecular Weight: 579.63



Procedure: A solution was prepared by adding sequentially 6-maleimido hexanoic acid (1 equivalent), (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) (TBTU, 1.2 equivalents) *N*,*N*-diisopropylethylamine (1.2 equivalent) in DMF (1 mL/mmol). After 30 minutes, compound **125** (1 equivalent) was added and the reaction mixture was stirred at 25 °C for 18 hours. The reaction mixture was diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated. The crude compound (as a yellow oil) was send to Nanotheters for the final purification.

^{1}H-NMR ($CDCl_3$): δ 1.48 – 1.63 (m, 3H), 2.46 (s, 5H), 2.54 – 2.63 (m, 3H), 3.46 (dt, J = 14.3, 6.2 Hz, 4H), 3.64 (dd, J = 9.4, 14.0 Hz, 7H), 6.60 (d, J = 6.6 Hz, 1H), 7.04 – 7.12 (m, 1H), 7.30 (ddd, J = 1.5, 6.3, 9.3 Hz, 1H), 7.37 – 7.43 (m, 1H), 7.53 (d, J = 2.7 Hz, 1H), 7.59 (d, J = 8.8 Hz, 1H), 7.90 – 8.03 (m, 1H), 8.34 (d, J = 8.7 Hz, 1H), 8.73 (d, J = 7.0 Hz, 1H), 11.14 (d, J = 8.7 Hz, 1H) ppm.

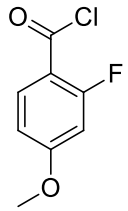
^{13}C-NMR ($CDCl_3$): δ 29.70 (CH_2, C-aliphatic), 35.86 (CH_2, C-aliphatic), 53.27 (CH_2, C-aliphatic), 53.55 (CH_2, C-aliphatic), 56.89 (CH_2, C-aliphatic), 60.40 (CH_2, C-aliphatic), 66.63 (CH_2, C-aliphatic), 66.81 (CH_2, C-aliphatic), 108.04 (CH, C-aliphatic), 112.33 (CH, C-aromatic), 117.49 (C, C-aromatic), 118.70 (CH, C-aromatic), 125.42 (CH, C-aromatic), 126.29 (CH, C-aromatic), 128.29 (CH, C-aromatic), 130.00 (C, C-aromatic), 132.12 (CH, C-aromatic), 142.52 (C, C-aromatic), 144.46 (C, C-aromatic), 145.28 (C, C-aromatic), 158.60 (C, C-aromatic), 159.35 (C, C-aromatic), 161.34 (C, C-aromatic), 161.71 (C, C-aromatic), 168.75 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.64 ppm.

MS(ESI)$^+$: 580.2 $[M + H]^+$

Synthesis of 2-fluoro-4-methoxybenzoyl chloride (230)

Chemical Formula: $C_8H_6ClFO_2$
Molecular Weight: 188.58



Procedure:

To a stirred solution of 2-fluoro-4-methoxybenzoic acid (1 equivalent) in thionyl chloride (10 equivalent) containing a drop of *N*,*N*- dimethyl formamide was heated at reflux temperature for 8 hours. The excess reagent was then removed under reduced pressure to give the crude compound as an orange powder.

Yield: -

State: orange powder

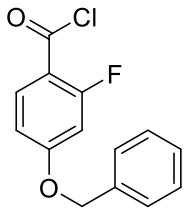
^{1}H-NMR ($CDCl_3$): δ 3.84 (s, 3H), 6.77 – 6.93 (m, 2H), 7.83 (t, *J* = 8.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.99 (CH_3, C-aliphatic), 102.24 (CH, C-aromatic), 102.45 (CH, C-aromatic), 110.59 (CH, C-aromatic), 133.27 (C, C-aromatic), 133.29 (C, C-aromatic), 164.61 (C, C-aromatic), 166.4 (C, C-aromatic) ppm.

Synthesis of 4-(benzyloxy)-2-fluorobenzoyl chloride (231)

Chemical Formula: $C_{14}H_{10}ClFO_2$

Molecular Weight: 264.68



Procedure:

To a stirred solution of 2-fluoro-4-benzyloxybenzoic acid (1 equivalent) in thionyl chloride (10 equivalent) containing a drop of *N*, *N*- dimethyl formamide was heated at 85 °C for 8 hours. The excess reagent was then removed under reduced pressure to give the crude compound as an orange powder, which was no further purified.

Yield: -

State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 4.97 – 5.27 (s, 2H), 6.13 (s, 2H), 6.74 – 6.90 (m, 2H), 7.43 – 7.47 (m, 2H), 7.97 – 8.14 (m, 2H) ppm.

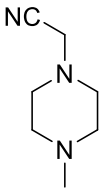
^{13}C-NMR ($CDCl_3$): δ 70.06 (CH_2, C-aliphatic), 103.07 (CH, C-aromatic, d, J = 26.25 Hz), 110.07 (C, C-aromatic), 111.09 (CH, C-aromatic), 127.21 (CH, C-aromatic), 127.53 (CH, C-aromatic), 128.26 (CH, C-aromatic), 128.69 (CH, C-aromatic), 128.83 (CH, C-aromatic), 134.27 (CH, C-aromatic), 135.09 (C, C-aromatic), 136.54 (CH, C-aromatic), 161.72 (C, C-aromatic), 163.38 (C, C-aromatic), 163.79(C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -103.54 ppm.

Synthesis of 2-(4-methylpiperazin-1-yl)acetonitrile (232)

Chemical Formula: $C_7H_{13}N_3$

Molecular Weight: 139.20



Procedure:

A solution of N-methyl piperazine (1 equivalent), chloroacetonitrile (1.2 equivalent) and potassium carbonate (5 equivalent) in acetonitrile (1 mL/mmol) was stirred for 48 hours at room temperature. The reaction mixture was filtered and the precipitate washed with *n*-hexane, the crude compound was collected as a brown powder. The crude compound was used for the next reduction reaction without further purification.

State: brown powder

Yield: -

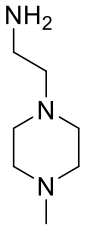
^{1}H-NMR ($CDCl_3$): δ 1.58 (s, 2H), 2.03 (s, 4H), 2.19 (s, 3H), 2.33 (d, J = 5.0 Hz, 1H), 2.51 (s, 1H), 2.66 (t, J = 4.7 Hz, 1H), 3.53 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 29.03 (CH_2, C-aliphatic), 44.21 (CH_2, C-aliphatic), 49.95 (CH_3, C-aliphatic), 56.6 (CH_2, C-aliphatic), 91.37 (C, C-aliphatic) ppm.

Synthesis of 2-(4-methylpiperazin-1-yl)ethan-1-amine (233)

Chemical Formula: $C_7H_{17}N_3$

Molecular Weight: 143.23



Procedure: A solution of compound **232** (1 equivalent) was dissolved in a 1:1 mixture of diethyl ether and tetrahydrofuran (1 mL/mmol) was added dropwise to a suspension of lithium aluminium hydride (2 equivalent) in diethyl ether (1 mL/mmol) at 0°C. The reaction mixture was stirred at 25 °C for 24 hours, cooled at 0 °C. Sodium hydroxide 6N was added, the solid was removed by filtration and the filtrate was concentrated under reduced pressure to give the titled product without further purification.

State: yellow powder

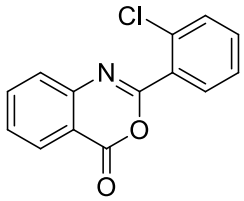
Yield: 39 %

^{1}H-NMR ($CDCl_3$): δ 1.68 (s, 4H), 2.30 (d, J = 6.0 Hz, 3H), 2.45 (t, J = 6.2 Hz, 4H), 2.56 (t, J = 46.6 Hz, 4H), 2.81 (t, J = 6.2 Hz, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 38.86 (CH_2, C-aliphatic), 46.70 (CH_3, C-aliphatic), 55.20 (CH_2, C-aliphatic), 56.41 (CH_2, C-aliphatic), 61.16 (CH_2, C-aliphatic) ppm.

Synthesis of 2-(2-chlorophenyl)-4H-benzo[d][1,3]oxazin-4-one (234)

Chemical Formula: $C_{14}H_8ClNO_2$
Molecular Weight: 257.67



Procedure: 1

State: white powder

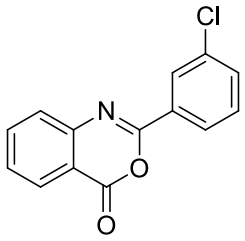
Yield: 89 %

^{1}H-NMR ($CDCl_3$): δ 7.57 (td, *J* = 7.6, 1.3 Hz, 1H), 7.62 – 7.74 (m, 3H), 7.76 (d, *J* = 7.8 Hz, 1H), 7.95 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.98 – 8.04 (m, 1H), 8.22 (dd, *J* = 1.2, 7.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 116.82 (C,C-aromatic), 127.11 (CH,C-aromatic), 127.49 (CH,C-aromatic), 128.05 (CH,C-aromatic), 129.38 (CH,C-aromatic),130.20 (C,C-aromatic), 130.64 (CH,C-aromatic), 131.67 (C,C-aromatic), 131.76 (CH,C-aromatic), 132.85 (CH,C-aromatic), 137.05 (CH,C-aromatic), 145.80 (C,C-aromatic), 155.78 (C,C-aromatic), 158.72 (C,C-aromatic) ppm.

Synthesis of 2-(3-chlorophenyl)-4H-benzo[d][1,3]oxazin-4-one (235)

Chemical formula: $C_{14}H_8ClNO_2$
Molecular weight: 257.67



State: white powder

Procedure: 1

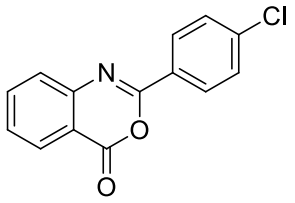
Yield: 39%

^{1}H-NMR ($CDCl_3$): δ 7.63-7.67 (m, 2H), 7.74 – 7.79 (m, 2H), 7.96 – 8.01 (m, 1H), 8.14 - 8.17 (m, 3H) ppm.

^{13}C-NMR ($CDCl_3$): δ 117.10 (C, C-aromatic), 126.42 (CH, C-aromatic), 127.05 (CH, C-aromatic), 127.20 (CH, C-aromatic), 128.11 (CH, C-aromatic), 128.96 (CH, C-aromatic), 131.06 (CH, C-aromatic), 132.23 (C, C-aromatic), 132.42 (CH, C-aromatic), 136.94 (CH, C-aromatic), 145.97 (C, C-aromatic), 155.33 (C, C-aromatic), 158.68 (C, C-aromatic), 159.1 (C, C-aromatic) ppm.

Synthesis of 2-(4-chlorophenyl)-4H-benzo[d][1,3]oxazin-4-one (236)

Chemical Formula: $C_{14}H_8ClNO_2$
Molecular Weight: 257.67



Procedure: 1

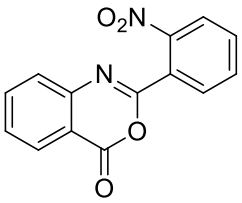
State: white powder

Yield: 45%

^{1}H-NMR ($CDCl_3$): δ 7.54 (m, 3H), 7.71 (d, *J* =5 Hz, 1H), 7.86 (m, 1H), 8.28 (m, 3H) ppm.

^{13}C-NMR ($CDCl_3$): δ 116.8 (C, C-aromatic), 124.1 (CH, C-aromatic), 126.5 (CH, C-aromatic), 127.9 (C, C-aromatic), 128.9 (CH, C-aromatic), 128.2 (CH, C-aromatic), 129.1 (CH, C-aromatic), 135.2 (CH, C-aromatic), 136.6 (C, C-aromatic), 146.1 (C, C-aromatic), 156.2 (C, C-aromatic), 159.4 (C, C-aromatic) ppm.

Synthesis of 2-(2-nitrophenyl)-4H-benzo[d][1,3]oxazin-4-one (237)
Chemical Formula: $C_{14}H_8N_2O_4$
Molecular Weight: 268.22



Procedure: 1

State: white powder

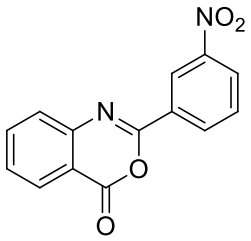
Yield: 71 %

^{1}H-NMR ($CDCl_3$): δ 7.61 – 7.65 (m, 1H). 7.69 – 7.72 (m, 1H), 7.70 - 7.78 (m, 2H), 7.84 – 7.93 (m, 1H), 8.01 – 8.04 (m, 1H), 8.08 (dd, *J* = 1.0, 8.1 Hz, 1H), 8.29 (dd, *J* = 1.2, 7.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 116.68 (C, C-aromatic), 124.57 (CH, C-aromatic), 124.98 (C, C-aromatic), 127.04 (CH, C-aromatic), 128.22 (CH, C-aromatic), 129.61 (CH, C-aromatic), 131.16 (CH, C-aromatic), 132.97 (CH, C-aromatic), 133.60 (CH, C-aromatic), 137.26 (CH, C-aromatic), 145.58 (C, C-aromatic), 148.15 (C, C-aromatic), 154.50 (C, C-aromatic), 158.20 (C, C-aromatic) ppm.

Synthesis of 2-(3-nitrophenyl)-4H-benzo[d][1,3]oxazin-4-one (238)

Chemical Formula: $C_{14}H_8N_2O_4$
Molecular Weight: 268.22



Procedure: 1

State: white powder

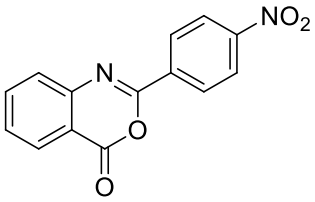
Yield: 61 %

^{1}H-NMR ($CDCl_3$): δ 7.62 (s, 1H), 7.77 (m, 2H), 7.96-7.89 (m, 1H), 8.30 (d, *J* = 1.2 Hz, 1H), 8.51-8.44 (m, 1H), 8.70 – 8.56 (m, 1H), 9.22 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 117.11 (C, C-aromatic), 123.39 (CH, C-aromatic), 126.88 (CH, C-aromatic), 128.87 (CH, C-aromatic), 129.17 (CH, C-aromatic), 129.98 (CH, C-aromatic), 132.19 (CH, C-aromatic), 131.65 (C, C-aromatic), 133.68 (CH, C-aromatic), 136.95 (CH, C-aromatic), 145.99 (C, C-aromatic), 158.36 (C, C-aromatic), 167.99 (C, C-aromatic), 173.58 (C, C-aromatic) ppm.

Synthesis of 2-(4-nitrophenyl)-4H-benzo[d][1,3]oxazin-4-one (239)

Chemical Formula: $C_{14}H_8N_2O_4$
Molecular Weight: 268.22



Procedure: 1

State: white powder

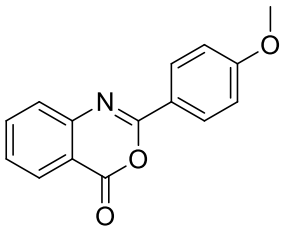
Yield: 95%

^{1}H-NMR ($CDCl_3$): δ 7.69 (t, *J* = 7.6 Hz, 2H), 7.79 (d, *J* =8.1Hz, 2H), 8.00 (t, *J* =7.8Hz, 2H), 8.19 (t, *J* = 7.8 Hz, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 117.20 (C, C-aromatic), 124.08 (CH, C-aromatic), 127.24 (CH, C-aromatic), 128.13 (CH, C-aromatic), 129.13 (CH, C-aromatic), 129.37 (CH, C-aromatic), 135.81 (C, C-aromatic), 136.98 (CH, C-aromatic), 145.77 (C, C-aromatic), 149.62 (C, C-aromatic), 154.74 (C, C-aromatic), 158.43 (C, C-aromatic),

Synthesis of 2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (240)

Chemical Formula: $C_{15}H_{11}NO_3$
Molecular Weight: 253.25



Procedure: 1

State: white powder

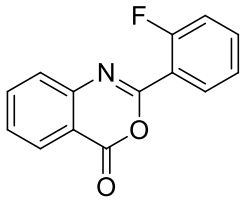
Yield: 95.31%

^{1}H-NMR ($CDCl_3$): δ 3.92 (s, 7H), 7.01 – 7.05 (m, 4H), 7.47 – 7.53 (m, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.80 – 7.85 (m, 2H), 8.25 (dd, *J* = 1.3, 7.9 Hz, 2H), 8.27 – 8.31 (m, 4H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.57 (CH_3, C-aliphatic), 114.16 (CH, C-aromatic), 116.73 (C, C-aromatic), 122.56 (C, C-aromatic), 126.93 (CH, C-aromatic), 127.72 (CH, C-aromatic), 128.57 (CH, C-aromatic), 130.30 (CH, C-aromatic), 132.85 (CH, C-aromatic), 136.52 (CH, C-aromatic), 147.37 (C, C-aromatic), 157.14 (C, C-aromatic), 159.82 (C, C-aromatic), 163.29 (C, C-aromatic)

Synthesis of 2-(2-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (241)

Chemical Formula: $C_{14}H_8FNO_2$
Molecular Weight: 241.22



Procedure: 1

State: yellow powder

Yield: 80%

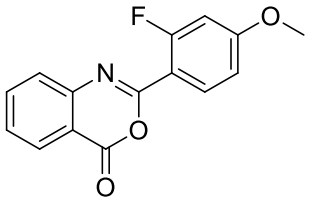
^{1}H-NMR ($CDCl_3$): δ 7.21 – 7.28 (m, 1H), 7.30 – 7.35 (m, 1H), 7.54 – 7.63 (m, 2H), 7.73 – 7.78 (m, 1H), 7.85 – 7.91 (m, 1H), 8.16 (td, *J* = 7.7, 1.7 Hz, 1H), 8.29 (dd, *J* = 7.9, 1.1 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 117.02 (C, C-aromatic),117.20 (CH, C-aromatic), 117.38 (CH, C-aromatic), 119.10 (C, C-aromatic), 124.33 (CH, C-aromatic), 127.46 (CH, C-aromatic), 128.60 (CH, C-aromatic), 128.80 (CH, C-aromatic), 131.16 (CH, C-aromatic), 133.99 (CH, C-aromatic), 134.06 (CH, C-aromatic), 136.65 (CH, C-aromatic), 146.71 (C, C-aromatic), 159.24 (C, C-aromatic), 160.37 (C, C-aromatic), 162.44 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 109.08 ppm.

Synthesis of 2-(2-fluoro-4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (242)

Chemical formula: $C_{15}H_{10}FNO_3$
Molecular weight: 271.24



Procedure: 1

State: white powder

Yield: 45%

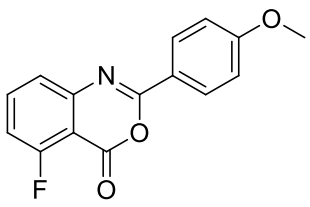
^{1}H-NMR ($CDCl_3$): δ 6.76 (dd, *J* = 2.4, 12.9 Hz, 1H), 6.84 (dd, *J* = 2.4, 8.9 Hz, 1H), 7.52 – 7.56 (m, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 7.82 – 7.88 (m, 1H), 8.13 (t, *J* = 8.7 Hz, 1H), 8.26 (dd, *J* = 1.2, 7.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.87 (CH_3, C-aliphatic), 102.66 (CH, C-aromatic), 110.68 (C, C-aromatic), 116.82 (C, C-aromatic), 127.19 (CH,C-aromatic), 128.21 (CH, C-aromatic), 128.52 (CH, C-aromatic), 132.17 (CH,C-aromatic), 132.19 (CH,C-aromatic), 136.51 (CH, C-aromatic), 147.08 (C, C-aromatic), 159.41 (C, C-aromatic), 161.80 (C, C-aromatic), 163.87 (C, C-aromatic), 164.34 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 105.53 ppm.

Synthesis of 5-fluoro-2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (243)

Chemical Formula: $C_{15}H_{10}FNO_3$
Molecular Weight: 271.24



Procedure: 1

State: white powder

Yield: 56 %

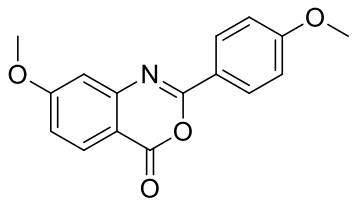
^{1}H-NMR ($CDCl_3$): δ 3.93 (s, 3H), 7.00 – 7.05 (m, 2H), 7.14 – 7.21 (m, 1H), 7.48 (d, *J* = 8.1 Hz, 1H), 7.72 – 7.78 (m, 1H), 8.25 – 8.32 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.57 (CH_3, C-aliphatic), 114.14 (CH, C-aromatic), 114.66 (CH, C-aromatic), 122.02 (C, C-aromatic), 122.79 (CH, C-aromatic), 130.53 (CH, C-aromatic), 132.85 (CH, C-aromatic), 134.99 (C, C-aromatic), 137.18 (CH, C-aromatic), 149.21 (C, C-aromatic), 160.89 (C, C-aromatic), 163.02 (C, C-aromatic), 163.6 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -106.84 ppm.

Synthesis of 7-methoxy-2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (244)

Chemical Formula: $C_{16}H_{13}NO_4$
Molecular Weight: 283.28



Procedure: 1

State: white powder

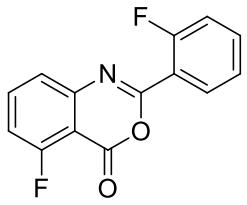
Yield: 90 %

^{1}H-NMR ($CDCl_3$): δ 3.92 (s, 3H), 3.97 (s, 3H), 6.99 – 7.05 (m, 3H), 7.08 (d, *J* = 2.4 Hz, 1H), 8.12 – 8.16 (m, 1H), 8.25 – 8.30 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.61 (CH_3, C-aliphatic), 55.88 (CH_3, C-aliphatic), 108.57 (CH, C-aromatic), 109.56 (C, C-aromatic), 114.14 (CH, C-aromatic), 116.91 (CH, C-aromatic), 122.63 (C, C-aromatic), 130.31 (CH, C-aromatic), 132.85 (CH, C-aromatic), 149.82 (C, C-aromatic), 155.00 (C, C-aromatic), 159.47 (C, C-aromatic), 163.30 (C, C-aromatic), 166.29 (C, C-aromatic) ppm.

Synthesis of 55-fluoro-2-(2-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (245)

Chemical Formula: $C_{14}H_7F_2NO_2$
Molecular Weight: 259.21



Procedure: 1

State: beige powder

Yield: 26 %

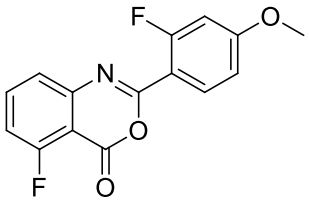
^{1}H-NMR ($CDCl_3$): δ 7.23 – 7.35 (m, 3H), 7.55 – 7.62 (m, 2H), 7.79 – 7.83 (m, 1H), 8.10 – 8.15 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 115.72 (CH, C-aromatic), 117.35 (CH, C-aromatic), 123.35 (CH, C-aromatic), 123.38 (CH, C-aromatic), 124.37 (C, C-aromatic), 124.40 (CH, C-aromatic), 131.20 (CH, C-aromatic), 134.36 (CH, C-aromatic), 134.44 (CH, C-aromatic), 137.32 (CH, C-aromatic), 137.40 (CH, C-aromatic), 148.42 (C, C-aromatic), 154.69 (CH, C-aromatic), 160.47 (CH, C-aromatic), 162.55 (C, C-aromatic), 162.89 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ- 106.22, - 108.66 ppm.

Synthesis of 5-fluoro-2-(2-fluoro-4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (246)

Chemical Formula: $C_{15}H_9F_2NO_3$
Molecular Weight: 289.23



Procedure: 1

State: white powder

Yield: 31 %

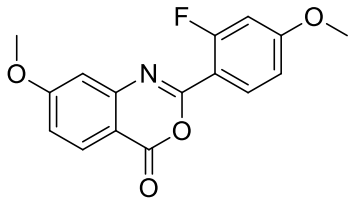
^{1}H-NMR ($CDCl_3$): δ 3.92 (s, 3H), 6.76 (dd, *J* = 2.4, 12.9 Hz, 1H), 6.82 – 6.85 (m, 1H), 7.21 (t, *J* = 8.7 Hz, 1H), 7.51 (t, *J* = 7.5 Hz, 1H), 7.72 – 7.80 (m, 1H), 8.13 (t, *J* = 8.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.94 (CH_3, C-aliphatic), 102.82 (CH, C-aromatic), 110.76 (CH, C-aromatic), 115.10 (C, C-aromatic), 123.08 (CH, C-aromatic), 132.27 (CH, C-aromatic), 137.18 (CH, C-aromatic), 137.26 (CH, C-aromatic), 148.84 (C, C-aromatic), 153.80 (C, C-aromatic), 160.78 (C, C-aromatic), 161.05 (C, C-aromatic), 161.97 (C, C-aromatic), 162.91 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -104.71, -106.43 ppm.

Synthesis of 2-(2-fluoro-4-methoxyphenyl)-7-methoxy-4H-benzo[d][1,3]oxazin-4-one (247)

Chemical Formula: $C_{16}H_{12}FNO_4$
Molecular Weight: 301.27



Procedure: 1

State: white powder

Yield: 17 %

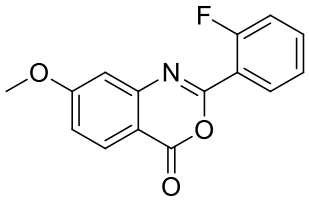
^{1}H-NMR ($CDCl_3$): δ 3.91 (s, 3H), 3.97 (s, 3H), 6.76 (dd, *J* = 2.2, 12.9 Hz, 1H), 6.84 (dd, *J* = 2.2, 8.9 Hz, 1H), 7.03 – 7.13 (m, 2H), 8.07 – 8.18 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.92 (CH_3, C-aliphatic), 102.88 (CH, C-aromatic), 106.3 (C, C-aromatic), 108.82 (CH, C-aromatic), 110.5 (C, C-aromatic), 110.63 (CH, C-aromatic), 117.49 (CH, C-aromatic), 130.16 (CH, C-aromatic), 132.19 (CH, C-aromatic), 154.9 (C, C-aromatic), 156.3 (C, C-aromatic), 159.4 (C, C-aromatic), 160.6 (C, C-aromatic), 162.1 (C, C-aromatic), 167.1(C, C-aromatic) ppm.

F-NMR ($CDCl_3$): δ -110.94 ppm.

Synthesis of 2-(2-fluorophenyl)-7-methoxy-4H-benzo[d][1,3]oxazin-4-one (248)

Chemical Formula: $C_{15}H_{10}FNO_3$
Molecular Weight: 271.24



Procedure: 1

State: beige powder

Yield: 54 %

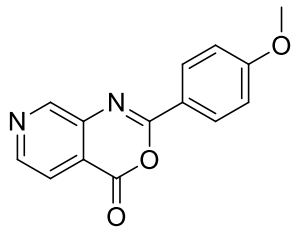
^{1}H-NMR ($CDCl_3$): δ 3.98 (s, 3H), 7.12 (dd, *J* = 2.5, 8.8 Hz, 1H), 7.16 (d, *J* = 2.4 Hz, 1H), 7.26 (dd, *J* = 8.4, 11.1 Hz, 1H), 7.32 (t, *J* = 7.6 Hz, 1H), 7.55 – 7.61 (m, 1H), 8.10 – 8.15 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.98 (CH_3, C-aliphatic), 106.3 (C, C-aromatic), 109.15 (CH, C-aromatic), 117.37 (CH, C-aromatic), 117.94 (CH, C-aromatic), 118.6 (C, C-aromatic), 124.32 (CH, C-aromatic), 130.23 (CH, C-aromatic), 131.14 (CH, C-aromatic), 133.97 (CH, C-aromatic), 154.9 (C, C-aromatic), 156.3 (C, C-aromatic), 159.1 (C, C-aromatic), 159.8 (C, C-aromatic), 167.6 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 107.60, - 109.22 ppm.

Synthesis of 2-(4-methoxyphenyl)-4H-pyrido[3,4-d][1,3]oxazin-4-one (249)

Chemical Formula: $C_{14}H_{10}N_2O_3$
Molecular Weight: 254.24



Procedure: 1

State: white powder

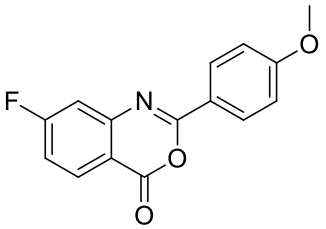
Yield: 75 %

^{1}H-NMR ($CDCl_3$): δ 3.93 (s, 3H), 7.02 – 7.08 (m, 2H), 8.00 (dd, *J* = 0.7, 5.1 Hz, 1H), 8.21 – 8.35 (m, 2H), 8.76 (d, *J* = 5.1 Hz, 1H), 9.11 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.60 (CH_3, C-aromatic), 114.14 (CH, C-aromatic), 114.36 (CH, C-aromatic), 119.96 (C, C-aromatic), 121.81 (C, C-aromatic), 122.33 (CH, C-aromatic), 130.61 (C, C-aromatic), 132.86 (C, C-aromatic), 147.82 (CH, C-aromatic), 150.24 (CH, C-aromatic), 158.06 (C, C-aromatic), 158.95 (C, C-aromatic), 163.84 (C, C-aromatic) ppm.

Synthesis of 7-fluoro-2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (250)

Chemical Formula: $C_{15}H_{10}FNO_3$
Molecular Weight: 271.24



Procedure: 1

State: white powder

Yield: 93 %

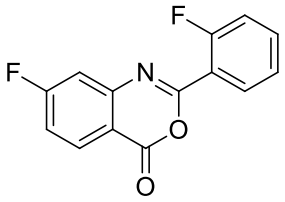
^{1}H-NMR ($CDCl_3$): δ 3.92 (s, 3H), 7.01 – 7.04 (m, 2H), 7.16 – 7.20 (m, 1H), 7.32 (dd, *J* = 2.4, 9.4 Hz, 1H), 8.23 – 8.26 (m, 1H), 8.26 – 8.29 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.56 (CH_3, C-aromatic), 112.83 (CH, C-aromatic), 113.01 (CH, C-aromatic), 114.24 (CH, C-aromatic), 116.04 (C, C-aromatic), 116.23 (CH, C-aromatic), 122.12 (CH, C-aromatic), 130.54 (C, C-aromatic), 131.35 (C, C-aromatic), 158.35 (C, C-aromatic), 158.88 (C, C-aromatic), 163.61 (C, C-aromatic), 168.84 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 99.60 ppm

Synthesis of 7-fluoro-2-(2-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (251)

Chemical Formula: $C_{14}H_7F_2NO_2$
Molecular Weight: 259.21



Procedure: 1

State: white powder

Yield: 94 %

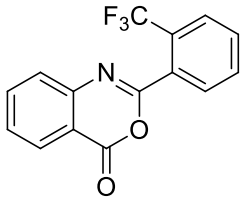
^{1}H-NMR ($CDCl_3$): δ 7.23 – 7.27 (m, 1H), 7.31 – 7.37 (m, 2H), 7.42 (dd, *J* = 1.24, 9.1 Hz, 1H), 7.57 – 7.63 (m, 1H), 8.11 – 8.16 (m, 1H), 8.31 (dd, *J* = 5.9, 8.8Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 113.50 (CH, C-aromatic), 113.61 (C, C-aromatic), 117.30 (CH, C-aromatic), 117.47 (CH, C-aromatic), 124.41 (CH, C-aromatic), 131.40 (CH, C-aromatic), 134.41 (CH, C-aromatic), 134.48 (CH, C-aromatic), 149.03 (C, C-aromatic), 158.29 (C, C-aromatic), 160.48 (C, C-aromatic), 162.56 (C, C-aromatic), 166.63 (C, C-aromatic), 168.87 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -104.11, -112.36 ppm.

Synthesis of 2-(2-(trifluoromethyl)phenyl)-4H-benzo[d][1,3]oxazin-4-one (252)

Chemical Formula: $C_{15}H_8F_3NO_2$
Molecular Weight: 291.22



Procedure: 1

State: white powder

Yield: 53 %

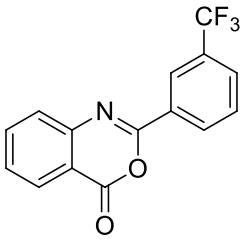
^{1}H-NMR ($CDCl_3$): δ 7.65 – 7.60 (m, 1H), 7.69 – 7.75 (m, 3H), 7.85 – 7.89 (m, 2H), 7.95 – 7.99 (m, 1H), 8.31 (dd, *J* = 1.4, 7.9 Hz, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ 116.91 (C, C-aromatic), 122.04 (C, C-aromatic), 127.46 (CH, C-aromatic), 128.69 (CH, C-aromatic), 129.12 (CH, C-aromatic), 129.21 (CH, C-aromatic), 130.09 (C, C-aromatic), 131.08 (CH, C-aromatic), 131.28 (CH, C-aromatic), 131.89 (CH, C-aromatic), 136.72 (CH, C-aromatic), 146.32 (C, C-aromatic), 156.80 (C, C-aromatic), 158.66 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -58.79 ppm.

Synthesis of 2-(3-(trifluoromethyl)phenyl)-4H-benzo[d][1,3]oxazin-4-one (253)

Chemical Formula: $C_{15}H_8F_3NO_2$
Molecular Weight: 291.22



Procedure: 1

State: white powder

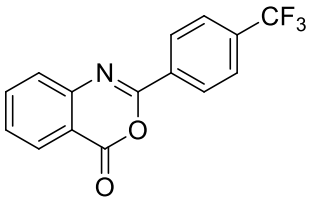
Yield: 41 %

^{1}H-NMR ($CDCl_3$): δ 7.60 (t, *J* = 7.6 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.76 (d, *J* = 8.1 Hz, 1H), 7.84 – 7.93 (m, 2H), 8.30 (d, *J* = 7.9 Hz, 1H), 8.53 (d, *J* = 7.9 Hz, 1H), 8.63 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$): δ 117.12 (C, C-aromatic), 125.29 (CH, C-aromatic), 127.44 (CH, C-aromatic), 128.82 (CH, C-aromatic), 129.80 (CH, C-aromatic), 131.23 (CH, C-aromatic), 131.34 (C, C-aromatic), 133.68 (CH, C-aromatic), 136.78 (CH, C-aromatic), 146.57 (C, C-aromatic), 155.60 (C, C-aromatic), 155.92 (C, C-aromatic), 159.05 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 62.75 ppm.

Synthesis of 2-(4-(trifluoromethyl)phenyl)-4H-benzo[d][1,3]oxazin-4-one (254)
Chemical Formula: $C_{15}H_8F_3NO_2$
Molecular Weight: 291.22



Procedure: 1

State: white powder

Yield: 73 %

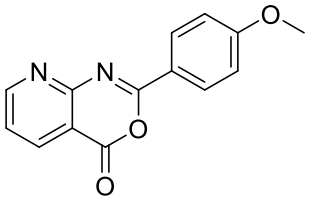
^{1}H-NMR ($CDCl_3$): δ 7.58 – 7.62 (m, 1H), 7.74 – 7.87 (m, 3H), 7.88 – 7.92 (m, 1H), 8.30 (dd, *J* = 1.4, 7.9 Hz, 1H), 8.47 (d, *J* = 8.2 Hz, 2H),

^{13}C-NMR ($CDCl_3$): δ 117.11 (C, C-aromatic), 125.47 (CH, C-aromatic), 127.64 (CH, C-aromatic), 128.65 (CH, C-aromatic), 128.77 (CH, C-aromatic), 128.95 (CH, C-aromatic), 136.64 (CH, C-aromatic), 133.43 (C, C-aromatic), 133.83 (C, C-aromatic), 134.14 (C, C-aromatic), 134,41 (C, C-aromatic), 146.58 (C, C-aromatic), 158.95 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -63.59 ppm.

Synthesis of 2-(4-methoxyphenyl)-4H-pyrido[2,3-d][1,3]oxazin-4-one (255)

Chemical Formula $C_{14}H_{10}N_2O_3$
Molecular Weight: 254.25



Procedure: 1

State: white powder

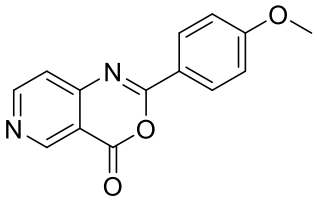
Yield: 43 %

^{1}H-NMR ($CDCl_3$): δ 3.93 (s, 3H), 7.05 (d, J = 8.9 Hz, 2H), 7.29 (s, 1H), 8.40 (d, J = 8.9 Hz, 2H), 8.56 (dd, J = 1.9, 7.8 Hz, 1H), 9.01 (dd, J = 4.6, 1.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.59 (CH_3, C-aromatic), 112.45 (C, C-aromatic), 114.34 (CH, C-aromatic), 121.68 (C, C-aromatic), 123.02 (CH, C-aromatic), 131.20 (CH, C-aromatic), 137.83 (CH, C-aromatic), 157.53 (CH, C-aromatic), 158.22 (C, C-aromatic), 159.64 (C, C-aromatic), 164.17 (C, C-aromatic) ppm.

Synthesis of 2-(4-methoxyphenyl)-4H-pyrido[4,3-d][1,3]oxazin-4-one (256)

Chemical Formula $C_{14}H_{10}N_2O_3$
Molecular Weight: 254.25



Procedure: 1

State: white powder

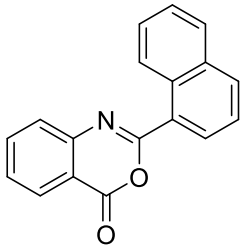
Yield: 76 %

^{1}H-NMR ($CDCl_3$): δ 3.94 (s, 3H), 6.97 – 7.10 (m, 2H), 7.29 (s, 1H), 8.28 – 8.43 (m, 2H), 8.93 (d, J = 5.6 Hz, 1H), 9.43 (d, *J* = 0.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.65 (CH_3, C-aromatic), 112.55 (C, C-aromatic), 114.41 (CH, C-aromatic), 120.14 (C, C-aromatic), 121.62 (CH, C-aromatic), 131.19 (CH, C-aromatic), 151.59 (CH, C-aromatic), 153.22 (CH, C-aromatic), 156.12 (C, C-aromatic), 158.15 (C, C-aromatic), 161.46 (C, C-aromatic), 164.30 (C, C-aromatic) ppm.

Synthesis of 2-(naphthalen-1-yl)-4H-benzo[d][1,3]oxazin-4-one (257)

Chemical Formula $C_{14}H_{10}N_2O_3$
Molecular Weight: 254.25



Procedure: 1

State: white powder

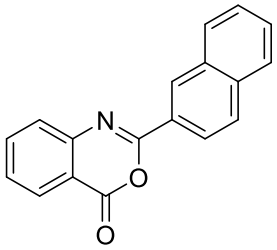
Yield: 89%

^{1}H-NMR ($CDCl_3$): δ 7.58 – 7.64 (m, 3H), 7.68 – 7.72 (m, 1H), 7.84 (dd, J = 0.6, 8.1 Hz, 1H), 7.89 – 7.94 (m, 1H), 7.97 (t, J = 6.3 Hz, 1H), 8.09 (d, J = 8.2 Hz, 1H), 8.32 – 8.38 (m, 2H), 9.17 (dd, J = 4.9, 8.3 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 117.03 (C, C-aromatic), 124.82 (CH, C-aromatic), 125.79 (CH, C-aromatic), 126.42 (CH, C-aromatic), 126.79 (C, C-aromatic), 127.44 (CH, C-aromatic), 127.88 (CH, C-aromatic), 128.57 (CH, C-aromatic), 128.63 (CH, C-aromatic), 128.85 (CH, C-aromatic), 130.04 (CH, C-aromatic), 130.78 (C, C-aromatic), 133.19 (CH, C-aromatic), 134.09 (C, C-aromatic), 136.31 (CH, C-aromatic), 146.85 (C, C-aromatic), 157.71 (C, C-aromatic), 159.77 (C, C-aromatic) ppm.

Synthesis of 2-(naphthalen-2-yl)-4H-benzo[d][1,3]oxazin-4-one (258)

Chemical Formula $C_{14}H_{10}N_2O_3$
Molecular Weight: 254.25



Procedure: 1

State: white powder

Yield: 91%

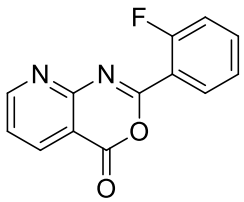
^{1}H-NMR ($CDCl_3$): δ 7.60 (dd, J = 1.2, 7.6 Hz, 3H), 7.77 (dd, J = 0.5, 8.1 Hz, 1H), 7.88 (dd, J = 7.3, 8.2, Hz, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.98 (d, J = 8.7 Hz, 1H), 8.03 (d, J = 7.9 Hz, 1H), 8.30 (dd, J = 1.1, 7.9 Hz, 1H), 8.40 (dd, J = 1.7, 8.7 Hz, 1H), 8.88 (s, 1H) ppm.

13C-NMR ($CDCl_3$): δ 117.09 (C, C-aromatic), 124.18 (CH, C-aromatic), 126.89 (CH, C-aromatic), 127.29 (C, C-aromatic), 127.45 (C, C-aromatic), 127.83 (CH, C-aromatic), 128.28 (CH, C-aromatic), 128.33 (CH, C-aromatic), 128.58 (CH, C-aromatic), 128.67 (CH, C-aromatic), 129.39 (CH, C-aromatic), 129.55 (CH, C-aromatic), 132.80 (C, C-aromatic), 135.37 (CH, C-aromatic), 136.61 (CH, C-aromatic), 147.13 (C, C-aromatic), 157.24 (C, C-aromatic), 159.68 (C, C-aromatic) ppm

Synthesis of 2-(2-fluorophenyl)-4H-pyrido[2,3-d][1,3]oxazin-4-one (259)

Chemical Formula: $C_{13}H_7FN_2O_2$

Molecular Weight: 242.21



Procedure: 1

State: white powder

Yield: 68%

^{1}H-NMR ($CDCl_3$): δ 7.23 – 7.28 (m, 1H), 7.31 – 7.37 (m, 1H), 7.56 (dd, J = 4.7, 4.7 Hz, 1H), 7.59 – 7.66 (m, 1H), 8.31 (td, J = 1.8, 7.7 Hz, 1H), 8.61 (dd, J = 2.0, 7.8 Hz, 1H), 9.07 (dd, J = 2.0, 4.7 Hz, 1H) ppm.

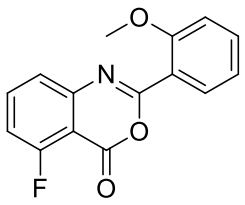
^{13}C-NMR ($CDCl_3$): δ 112.89 (C, C-aromatic), 117.39 (CH, C-aromatic), 117.57 (CH, C-aromatic), 124.06 (CH, C-aromatic), 124.43 (CH, C-aromatic), 131.79 (CH, C-aromatic), 135.07 (CH, C-aromatic), 135.15 (C, C-aromatic), 137.90 (CH, C-aromatic), 157.53 (C, C-aromatic), 157.66 (CH, C-aromatic), 159.11 (C, C-aromatic), 160.78 (C, C-aromatic), 162.87 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -107.15 ppm.

Synthesis of 5-fluoro-2-(2-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (260)

Chemical Formula: $C_{15}H_{10}FNO_3$

Molecular Weight: 271.24



Procedure: 1

State: white powder

Yield: 98%

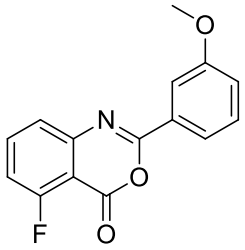
^{1}H-NMR ($CDCl_3$): δ 3.96 (s, 3H), 7.10 (t, *J* = 8.5 Hz, 1H), 7.21 (dd, *J* = 8.5, 9.5 Hz, 1H), 7.44 (t, *J* = 8.6 Hz, 1H), 7.51 – 7.55 (m, 1H), 7.74 – 7.79 (m, 1H), 7.82 (d, *J* = 5.5 Hz, 1H), 7.92 (d, *J* = 8.1 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.13 (CH_3, C-aliphatic), 112.22 (CH, C-aromatic), 115.15 (CH, C-aromatic, *J* = 26.25 Hz), 120.09 (CH, C-aromatic), 120.61 (C, C-aromatic), 123.11 (CH, C-aromatic), 123.15 (CH, C-aromatic), 131.39 (CH, C-aromatic), 133.54 (CH, C-aromatic), 137.08 (CH, C-aromatic), 148.87 (C, C-aromatic), 155.35 (C, C-aromatic), 158.71 (C, C-aromatic), 158.80 (C, C-aromatic), 160.76 (C, C-aromatic), 162.89 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -106.66 ppm.

Synthesis of 5-fluoro-2-(3-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (261)
Chemical Formula: $C_{15}H_{10}FNO_3$

Molecular Weight: 271.24



Procedure: 1

State: white powder

Yield: 94%

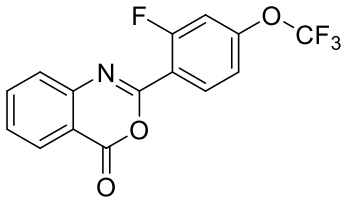
^{1}H-NMR ($CDCl_3$): δ 3.94 (s, 3H), 7.16 (dd, *J* = 2.6, 8.2 Hz, 1H), 7.21 (dd, *J* = 8.4, 9.5 Hz, 1H), 7.44 (t, *J* = 8.0 Hz, 1H), 7.53 (d, *J* = 8.1 Hz, 1H), 7.77 – 7.81 (m, 1H), 7.82 (dt, *J* = 2.8, 5.4 Hz, 1H), 7.92 (dd, *J* = 1.4, 7.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.59 (CH_3, C-aliphatic), 112.75 (CH, C-aromatic), 115.11 (CH, C-aromatic), 119.71 (CH, C-aromatic), 121.06 (CH, C-aromatic), 123.12 (CH, C-aromatic), 129.83 (CH, C-aromatic), 131.09 (CH, C-aromatic), 137.18 (C, C-aromatic), 137.26 (CH, C-aromatic), 148.77 (C, C-aromatic), 155.22 (C, C-aromatic), 157.75 (C, C-aromatic), 159.93 (C, C-aromatic), 160.87 (C, C-aromatic), 163.00 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -106.52 ppm.

Synthesis of 2-(2-fluoro-4-(trifluoromethoxy)phenyl)-4H-benzo[d][1,3]oxazin-4-one (262)
Chemical Formula: $C_{15}H_7F_4NO_3$

Molecular Weight: 325.22



Procedure: 1

State: white powder

Yield: 95%

^{1}H-NMR ($CDCl_3$): δ 7.11 – 7.21 (m, 2H), 7.58 – 7.63 (m, 1H), 7.75 (d, J = 8.1 Hz, 1H), 7.86 – 7.92 (m, 1H), 8.24 (t, J = 8.5 Hz, 1H), 8.29 (dd, J = 1.2, 7.9 Hz, 1H) ppm.

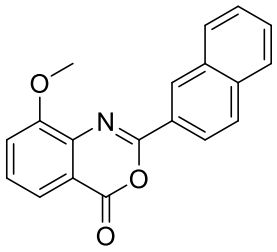
^{13}C-NMR ($CDCl_3$): δ 109.97 (CH, C-aromatic), 116.14 (CH, C-aromatic), 117.03 (C, C-aromatic), 119.00 (C, C-aromatic), 117.2 (C, C-aromatic), 127.51 (CH, C-aromatic), 128.68 (CH, C-aromatic), 129.01 (CH, C-aromatic), 132.47 (CH, C-aromatic), 136.72 (CH, C-aromatic), 146.27 (C, C-aromatic), 152.46 (C, C-aromatic), 158.85 (C, C-aromatic), 160.68 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 57.99, -103.77 ppm.

Synthesis of 8-methoxy-2-(naphthalen-2-yl)-4H-benzo[d][1,3]oxazin-4-one (263)

Chemical Formula: $C_{19}H_{13}NO_3$

Molecular Weight: 303.1



Procedure: 1

State: white powder

Yield: 89%

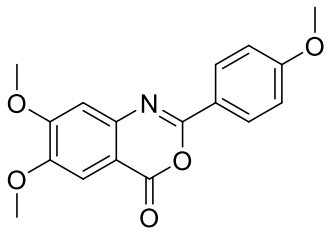
^{1}H-NMR ($CDCl_3$): δ 4.11 (s, 3H), 7.34 – 7.39 (m, 1H), 7.51 (t, J = 7.9 Hz, 1H), 7.57 – 7.69 (m, 2H), 7.87 – 7.94 (m, 2H), 7.97 (d, J = 8.2 Hz, 1H), 8.03 (d, J = 7.9 Hz, 1H), 8.44 (dd, J = 1.7, 8.7 Hz, 1H), 8.89 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.64 (CH3, C-aliphatic), 117.31 (CH, C-aromatic), 118.03 (C, C-aromatic), 119.91 (CH, C-aromatic), 124.36 (CH, C-aromatic), 126.85 (CH, C-aromatic), 127.46 (CH, C-aromatic), 127.83 (CH, C-aromatic), 128.3 (CH, C-aromatic), 128.54 (C, C-aromatic), 128.7 (CH, C-aromatic), 129.42 (CH, C-aromatic), 129.57 (CH, C-aromatic) 132.76 (C, C-aromatic), 135.35 (C, C-aromatic), 137.09 (C, C-aromatic), 154.37 (C, C-aromatic), 156.65 (C, C-aromatic), 159.63 (C, C-aromatic) ppm.

Synthesis of 6, 7-dimethoxy-2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (264)

Chemical Formula: $C_{17}H_{15}NO_5$

Molecular Weight: 313.10



Procedure: 1

State: white powder

Yield: 72%

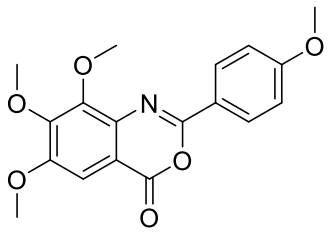
^{1}H-NMR ($CDCl_3$): δ 3.83 (s, 3H), 3.93 (s, 3H), 3.96 (s, 3H), 6.89 – 6.97 (m, 2H), 7.01 (s, 1H), 7.19 (s, 1H), 7.48 (s, 1H), 8.14 – 8.18 (m, 1H),

^{13}C-NMR ($CDCl_3$): δ 55.51 (CH3, C-aliphatic), 56.44 (CH3, C-aliphatic), 56.50 (CH3, C-aliphatic), 107.71 (CH, C-aromatic), 107.85 (CH, C-aromatic), 107.56 (CH, C-aromatic), 109.26 (C, C-aromatic), 114.15 (CH, C-aromatic), 122.80 (C, C-aromatic), 129.93 (CH, C-aromatic), 132.85 (CH, C-aromatic), 143.76 (C, C-aromatic), 149.37 (C, C-aromatic), 156.49 (C, C-aromatic) 156.72 (C, C-aromatic), 159.74 (C, C-aromatic), 163.05 (C, C-aromatic) ppm.

Synthesis of 6, 7, 8-trimethoxy-2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (265)

Chemical Formula: $C_{18}H_{17}NO_6$

Molecular Weight: 343.34



Procedure: 1

State: white powder

Yield: 79%

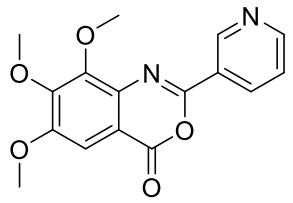
^{1}H-NMR ($CDCl_3$): δ3.92 (s, 3H), 3.99 (s, 3H), 4.09 (s, 3H), 4.18 (s, 3H), 6.96 – 7.06 (m, 2H), 7.45 (s, 1H), 8.09 – 8.15 (m, 1H), 8.26 – 8.33 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.51 (CH_3, C-aliphatic), 56.40 (CH_3, C-aliphatic), 61.51 (CH_3, C-aliphatic), 62.71 (CH_3, C-aliphatic), 104.02 (CH, C-aromatic), 112.06 (C, C-aromatic), 114.11 (CH, C-aromatic), 122.83 (C, C-aromatic), 129.99 (CH, C-aromatic), 136.92 (C, C-aromatic), 147.64 (C, C-aromatic), 149.45 (C, C-aromatic), 152.99 (C, C-aromatic), 155.26 (C, C-aromatic), 159.76 (C, C-aromatic), 163.05 (C, C-aromatic) ppm.

Synthesis of 6,7,8-trimethoxy-2-(pyridin-3-yl)-4H-benzo[d][1,3]oxazin-4-one (266)

Chemical Formula: $C_{18}H_{17}NO_6$

Molecular Weight: 343.34



Procedure: 1

State: white powder

Yield: 75%

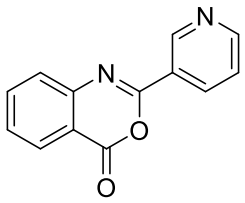
^{1}H-NMR ($CDCl_3$): δ 4.02 (s, 3H), 4.10 (s, 3H), 4.20 (s, 3H), 7.29 (s, 1H), 7.37 – 7.59 (m, 1H), 8.50 – 8.65 (m, 1H), 8.81 (dd, J = 1.7, 4.8 Hz, 1H), 9.55 (dd, J = 0.7, 2.2 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.49 (CH_3, C-aromatic), 61.56 (CH_3, C-aromatic), 62.87 (CH_3, C-aromatic), 104.24 (CH, C-aromatic), 112.54 (C, C-aromatic), 123.45 (CH, C-aromatic), 126.60 (C, C-aromatic), 135.14 (CH, C-aromatic), 135.95 (C, C-aromatic), 148.21 (C, C-aromatic), 148.52 (C, C-aromatic), 149.47 (CH, C-aromatic), 149.62 (C, C-aromatic), 152.68 (CH, C-aromatic), 153.88 (C, C-aromatic), 158.94 (C, C-aromatic) ppm.

Synthesis of 2-(pyridin-3-yl)-4H-benzo[d][1,3]oxazin-4-one (267)

Chemical Formula: $C_{13}H_8N_2O_2$

Molecular Weight: 224.22



Procedure: 1

State: white powder

Yield: 81%

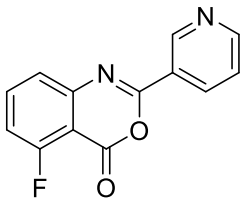
^{1}H-NMR ($CDCl_3$): δ 7.49 (dd, J = 4.8, 8.0 Hz, 1H), 7.60 (td, J = 1.1, 7.9 Hz, 1H), 7.76 (dd, J = 0.6, 8.1 Hz, 1H), 7.90 (ddd, J = 1.5, 7.4, 8.1 Hz, 1H), 8.30 (dd, J = 1.3, 7.9 Hz, 1H), 8.46 – 8.63 (m, 1H), 8.83 (dd, J = 1.7, 4.8 Hz, 1H), 9.55 (dd, J = 0.7, 2.2 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 117.20 (C, C-aromatic), 123.48 (C, C-aromatic), 126.42 (CH, C-aromatic), 127.42 (C, C-aromatic), 128.78 (CH, C-aromatic), 128.86 (CH, C-aromatic), 135.49 (CH, C-aromatic), 136.81 (CH, C-aromatic), 146.52 (C, C-aromatic), 149.73 (C, C-aromatic), 152.99 (C, C-aromatic), 155.60 (C, C-aromatic), 158.98 (C, C-aromatic) ppm.

Synthesis of 5-fluoro-2-(pyridin-3-yl)-4H-benzo[d][1,3] oxazin-4-one (268)

Molecular Formula: $C_{13}H_7FN_2O_2$

Molecular Weight: 242.21



Procedure: 1

Yield: 62%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 7.22 – 7.29 (m, 2H), 7.50 (dd, J = 4.8, 8.0 Hz, 1H), 7.57 (d, J = 8.1 Hz, 1H), 7.85 (td, J = 5.5, 8.2 Hz, 1H), 8.36 – 8.65 (m, 1H), 8.84 (dd, J = 1.7, 4.8 Hz, 1H), 9.45 – 9.52 (m, 1H) ppm.

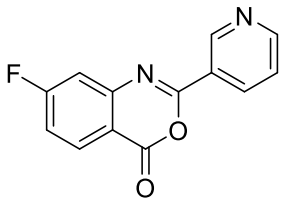
^{13}C-NMR ($CDCl_3$): δ 115.75-115.92 (CH, C-aromatic), 123.30 (CH, C-aromatic), 123.33 (CH, C-aromatic), 125.97 (C, C-aromatic), 135.62 (CH, C-aromatic), 137.51-137.59 (CH, C-aromatic), 148.21 (C, C-aromatic), 149.86 (CH, C-aromatic), 153.30 (CH, C-aromatic), 154.46 (C, C-aromatic), 156.36 (C, C-aromatic), 160.87 (C, C-aromatic), 163.01 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -105.84 ppm.

Synthesis of 7-fluoro-2-(pyridin-3-yl)-4H-benzo[d][1,3]oxazin-4-one) (269)

Molecular Formula: $C_{13}H_7FN_2O_2$

Molecular Weight: 242.205



Procedure: 1

Yield: 58%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 7.27 – 7.35 (m, 1H), 7.42 (dd, J = 2.4, 9.1 Hz, 1H), 7.50 (dd, J = 4.8, 8.0 Hz, 1H), 8.31 (dd, J = 5.9, 8.7 Hz, 1H), 8. 55– 8.60 (m, 1H), 8.85 (dd, J = 1.7, 4.8 Hz, 1H), 9.54 (d, J = 1.5 Hz, 1H) ppm.

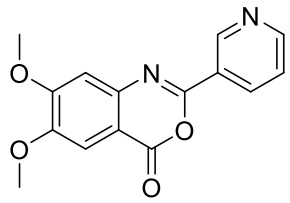
^{13}C-NMR ($CDCl_3$): δ 113.57 (CH, C-aromatic), 113.73 (C, C-aromatic), 117.27 (CH, C-aromatic), 123.52 (CH, C-aromatic), 126.07 (C, C-aromatic), 131.59 (CH, C-aromatic), 135.66 (CH, C-aromatic), 149.88 (CH, C-aromatic), 153.34 (CH, C-aromatic), 156.73 (C, C-aromatic), 158.00 (C, C-aromatic), 166.86 (C, C-aromatic), 168.91 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -98.26 ppm.

Synthesis of 6, 7-dimethoxy-2-(pyridin-3-yl)-4H-benzo[d][1,3] oxazin-4-one (270)

Molecular Formula: $C_{15}H_{12}N_2O_4$

Molecular Weight: 284.27



Procedure: 1

Yield: 58 %

State: white powder

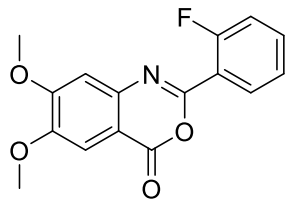
^{1}H-NMR ($CDCl_3$): δ 4.04 (s, 3H), 4.08 (s, 3H), 7.04 (bs, 1H), 7.37– 7.64 (m, 1H), 7.55 (d, J = 56.3 Hz, 1H), 8.31 – 8.61 (m, 1H), 8.81 (dd, J = 1.7, 4.8, Hz, 1H), 9.52 (d, J = 1.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.52 (CH_3, C-aliphatic), 56.60 (CH_3, C-aliphatic), 107.72 (CH, C-aromatic), 108.26 (CH, C-aromatic), 109.84 (C, C-aromatic), 123.45 (CH, C-aromatic), 126.60 (C, C-aromatic), 135.12 (CH, C-aromatic), 142.88 (C, C-aromatic), 149.46 (CH, C-aromatic), 150.15 (C, C-aromatic), 152.66 (CH, C-aromatic), 154.81 (C, C-aromatic), 156.63 (C, C-aromatic), 158.96 (C, C-aromatic) ppm.

Synthesis of 2-(2-fluorophenyl)-6, 7-dimethoxy-4H-benzo[d][1,3]oxazin-4-one (271)

Molecular Formula: $C_{16}H_{12}NO_4$

Molecular Weight: 301.27



Procedure: 1

Yield: 78 %

State: white powder

^{1}H-NMR ($CDCl_3$): δ 3.95 (s, 3H), 3.97 (s, 3H), 7.08 (s, 1H), 7.11 – 7.25 (m, 2H), 7.39 – 7.49 (m, 1H), 7.49 – 7.56 (m, 1H), 8.04 (td, J = 1.8, 7.7 Hz, 1H) ppm.

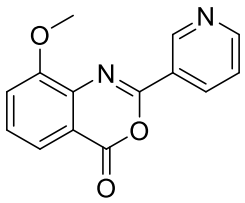
^{13}C-NMR ($CDCl_3$): δ 56.50 (CH_3, C-aliphatic), 56.59 (CH_3, C-aliphatic), 107.54 (CH, C-aromatic), 108.38 (CH, C-aromatic), 109.66 (C, C-aromatic), 117.16 (CH, C-aromatic), 124.32 (CH, C-aromatic), 130.91 (CH, C-aromatic), 133.65 (CH, C-aromatic), 143.04 (C, C-aromatic), 150.08 (C, C-aromatic), 154.10 (C, C-aromatic), 156.49 (C, C-aromatic), 159.18 (C, C-aromatic), 160.23 (C, C-aromatic), 162.29 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.78 ppm.

Synthesis of 8-methoxy-2-(pyridin-3-yl)-4H-benzo[d][1,3]oxazin-4-one (272)

Molecular Formula: $C_{14}H_{10}N_2O_3$

Molecular Weight: 254.25



Procedure: 1

Yield: 72 %

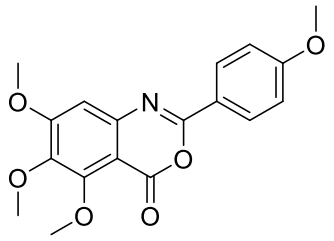
State: white powder

^{1}H-NMR ($CDCl_3$): δ 3.99 (s, 3H), 7.19 (s, 1H), 7.35 – 7.43 (m, 1H), 7.44 (t, J = 8.0 Hz, 1H), 7.78 (dd, J = 1.2, 7.9 Hz, 1H), 8.43 – 8.64 (m, 1H), 8.72 (dd, J = 1.7, 4.8 Hz, 1H), 9.46 (dd, J = 0.7, 2.2 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.66 (CH_3, C-aliphatic), 117.65 (CH, C-aromatic), 118.19 (C, C-aromatic), 119.97 (CH, C-aromatic), 123.43 (CH, C-aromatic), 126.53 (C, C-aromatic), 129.31 (CH, C-aromatic), 135.60 (CH, C-aromatic), 136.46 (C, C-aromatic), 149.69 (CH, C-aromatic), 152.92 (CH, C-aromatic), 154.50 (C, C-aromatic), 158.89 (CH, C-aromatic) ppm.

Synthesis of 5, 6, 7-trimethoxy-2-(4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (**273**)

Chemical Formula: $C_{18}H_{17}NO_6$
Molecular Weight: 343.33



Procedure: 1

Yield: 93 %

State: white solid

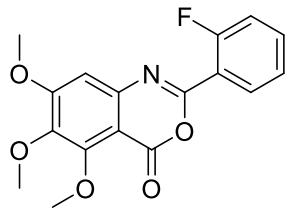
^{1}H-NMR ($CDCl_3$): δ 3.92 (s, 3H), 3.99 (s, 3H), 4.1 (s, 3H), 4.19 (s, 3H), 7.1 (d, J =9.05 Hz, 2H), 7.45 (s, 1H), 8.28 (d, J =8.9 Hz, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.52 (CH_3, C-aliphatic), 56.40 (CH_3, C-aliphatic), 61.53 (CH_3, C-aliphatic), 62, 73 (CH_3, C-aliphatic), 103.99 (CH, C-aromatic), 112.04 (C, C-aromatic), 114.10 (CH, C-aromatic), 122.79 (C, C-aromatic), 129.98 (CH, C-aromatic), 136.92 (C, C-aromatic), 147.61 (C, C-aromatic), 149.42 (C, C-aromatic), 152.97 (C, C-aromatic), 155.24 (C, C-aromatic), 159.77 (C, C-aromatic), 163.04 (C, C-aromatic) ppm.

Synthesis of 2-(2-fluorophenyl)-5, 6, 7-trimethoxy-4H-benzo[d][1,3]oxazin-4-one (274)

Chemical Formula: $C_{17}H_{14}FNO_5$

Molecular Weight: 331.30



Procedure: 1

Yield: 69 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 4.00 (s, 3H), 4.09 (s, 3H), 4.20 (s, 3H), 7.21-7.25 (m, 1H), 7.3 (td, J =1.2, 7.5 Hz, 1H), 7.47 (s, 1H), 7.52-7.57 (m, 2H), 8.16 (td, J =1.65, 7.7 Hz, 1H) ppm.

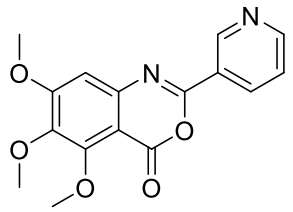
^{13}C-NMR ($CDCl_3$): δ 56.46 (CH_3, C-aliphatic), 61.56 (CH_3, C-aliphatic), 62.78 (CH_3, C-aliphatic), 103.86 (CH, C-aromatic), 112.36 (C, C-aromatic), 117.26 (CH, C-aromatic), 119.13 (C, C-aromatic), 124.25 (CH, C-aromatic), 130.86 (CH, C-aromatic), 133.66 (CH, C-aromatic), 136.06 (C, C-aromatic), 148.03 (C, C-aromatic), 149.31 (C, C-aromatic), 153.74 (C, C-aromatic), 159.25 (C, C-aromatic), 160.41 (C, C-aromatic), 162.49 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 102.27 ppm.

Synthesis of 5, 6, 7-trimethoxy-2-(pyridin-3-yl)-4H-benzo[d][1,3]oxazin-4-one (275)

Chemical Formula: $C_{16}H_{14}N_2O_5$

Molecular Weight: 314.29



Procedure: 1

Yield: 50 %

State: white solid

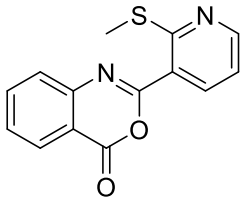
^{1}H-NMR ($CDCl_3$): δ 4.01 (s, 3H), 4.09 (s, 3H), 4.19 (s, 3H), 7.47 (s, 1H), 7.49 (dd, J = 0.8, 7.55 Hz, 1 H), 8.58 (dt, J =1.9, 8.1 Hz, 1H), 8.80 (dd, J =1.7, 4.75 Hz, 1H), 9.54 (d, J =1.95 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.49 (CH_3, C-aliphatic), 61.57 (CH_3, C-aliphatic), 62.89 (CH_3, C-aliphatic), 104.22 (CH, C-aromatic), 112.52 (C, C-aromatic), 123.58 (CH, C-aromatic), 126.72 (C, C-aromatic), 135.39 (CH, C-aromatic), 135.90 (C, C-aromatic), 148.02 (C, C-aromatic), 149.17 (CH, C-aromatic), 149.61 (C, C-aromatic), 152.35 (CH, C-aromatic), 153.23 (C, C-aromatic), 153.89 (C, C-aromatic), 158.89 (C, C-aromatic) ppm.

Synthesis of 2-(2-(methylthio)pyridin-3-yl)-4H-benzo[d][1,3]oxazin-4-one (276)

Chemical Formula: $C_{14}H_{10}N_2O_2S$

Molecular Weight: 270.05



Procedure: 1

Yield: 85 %

State: white solid

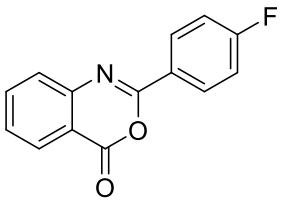
^{1}H-NMR ($CDCl_3$): δ 2.62 (s, 3H), 7.18 (dd, J = 4.7, 7.9 Hz, 1H), 7.49 – 7.66 (m, 1H), 7.83 (dd, J = 0.7, 8.1 Hz, 1H), 7.86 – 7.93 (m, 1H), 8.28 (dd, J = 1.1, 7.9 Hz, 1H), 8.42 (dd, J = 1.8, 7.9 Hz, 1H), 8.64 (dd, J = 1.8, 4.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 14.73 (CH_3, C-aliphatic), 116.89 (C, C-aromatic), 118.22 (CH, C-aromatic), 123.64 (C, C-aromatic), 127.32 (CH, C-aromatic), 128.66 (CH, C-aromatic), 128.87 (CH, C-aromatic), 136.72 (CH, C-aromatic), 137.23 (C, C-aromatic), 146.20 (CH, C-aromatic), 151.14, 154.78 (C, C-aromatic), 159.32 (C, C-aromatic), 161.86 (C, C-aromatic) ppm.

Synthesis of 2-(4-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (277)

Chemical Formula: $C_{14}H_8FNO_2$

Molecular Weight: 241.22



Procedure: 1

Yield: 95 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 7.19 – 7.26 (m, 2H), 7.53 – 7.59 (m, 1H), 7.71 (d, J = 8.1 Hz, 1H), 7.83 – 7.90 (m, 1H), 8.27 (dd, J = 1.2, 7.9 Hz, 1H), 8.33 – 8.40 (m, 2H) ppm.

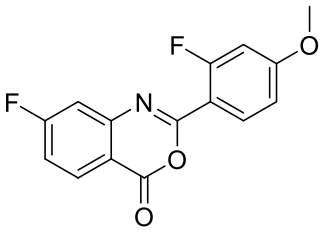
^{13}C-NMR ($CDCl_3$): δ 115.91 (C, C-aromatic), 116.09 (C, C-aromatic), 116.88 (CH, C-aromatic), 126.47 (CH, C-aromatic), 127.16 (CH, C-aromatic), 128.29 (CH, C-aromatic), 128.65 (CH, C-aromatic), 130.71 (CH, C-aromatic), 130.78 (CH, C-aromatic), 136.64 (CH, C-aromatic), 146.93 (C, C-aromatic), 156.33 (C, C-aromatic), 159.38 (C, C-aromatic), 164.60 (C, C-aromatic), 166.63 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -105.99 ppm.

Synthesis of 7-fluoro-2-(2-fluoro-4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (278)

Chemical Formula: $C_{15}H_9F_2NO_3$

Molecular Weight: 289.24



Procedure: 1

Yield: 95 %

State: white solid

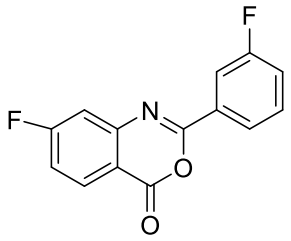
^{1}H-NMR ($CDCl_3$): δ 3.82 (s, 3H), 6.60 – 6.72 (m, 1H), 6.75 (dd, J = 4.5, 6.9 Hz, 1H), 7.09 – 7.16 (m, 1H), 7.26 (dt, J = 10.8, 21.6 Hz, 1H), 8.03 (t, J = 8.7 Hz, 1H), 8.15 – 8.25 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.93 (CH_3, C-aliphatic), 102.92 (CH, C-aromatic), 110.79 (CH, C-aromatic), 113.15 (CH, C-aromatic), 116.72 (CH, C-aromatic), 131.36 (CH, C-aromatic), 132.31 (CH, C-aromatic), 149.55 (C, C-aromatic), 149.66 (C, C-aromatic), 158.50 (C, C-aromatic), 161.98 (C, C-aromatic), 164.06 (C, C-aromatic), 164.69 (C, C-aromatic), 166.76 (C, C-aromatic), 168.81 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 98.82, 104.53 ppm.

Synthesis of 7-fluoro-2-(3-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (279)

Chemical Formula: $C_{14}H_7F_2NO_2$
Molecular Weight: 259.21



Procedure: 1

Yield: 92 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 7.14 – 7.26 (m, 2H), 7.28 (dd, J = 2.5, 9.1 Hz, 1H), 7.42 (td, J = 5.6, 8.1 Hz, 1H), 7.92 (dd, J = 2.5, 9.6 Hz, 1H), 8.04 – 8.04 (m, 1H), 8.19 (dd, J = 5.9, 8.7 Hz, 1H) ppm.

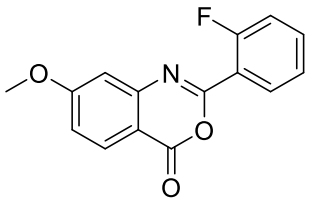
^{13}C-NMR ($CDCl_3$): δ 113.37 (CH, C-aromatic), 115.31 (CH, C-aromatic), 116.95 (CH, C-aromatic), 119.98 (CH, C-aromatic), 124.18 (CH, C-aromatic), 130.45 (CH, C-aromatic), 131.45 (CH, C-aromatic), 132.06 (C, C-aromatic), 149.16 (C, C-aromatic), 158.21 (C, C-aromatic), 161.89 (C, C-aromatic), 163.86 (C, C-aromatic), 166.81 (C, C-aromatic), 168.86 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -98.44, -111.32 ppm.

Synthesis of 2-(2-fluorophenyl)-7-methoxy-4H-benzo[d][1,3]oxazin-4-one (280)

Chemical Formula: $C_{15}H_{10}FNO_3$

Molecular Weight: 271.25



Procedure: 1

Yield: 89 %

State: white solid

^{1}H-NMR ($CDCl_3$): δ 4.06 (s, 3H), 7.20 – 7.37 (m, 3H), 7.49 – 7.61 (m, 2H), 7.87 (dd, J = 1.2, 7.9 Hz, 1H), 8.16 (td, J = 1.8, 7.6 Hz, 1H) ppm.

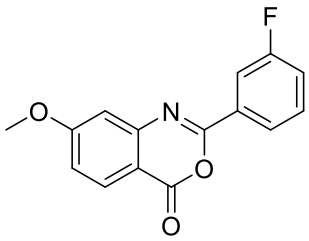
^{13}C-NMR ($CDCl_3$): δ 56.65 (CH_3, C-aromatic), 117.12 (CH, C-aromatic), 117.19 (CH, C-aromatic), 117.38 (C, C-aromatic), 117.98 (C, C-aromatic), 119.73 (CH, C-aromatic), 124.30 (CH, C-aromatic), 129.23 (CH, C-aromatic), 131.23 (CH, C-aromatic), 133.90 (CH, C-aromatic), 136.62 (C, C-aromatic), 154.45 (C, C-aromatic), 159.16 (C, C-aromatic), 160.39 (C, C-aromatic), 162.46 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$):δ -108.94 ppm.

Synthesis of 2-(3-fluorophenyl)-7-methoxy-4H-benzo[d][1,3]oxazin-4-one (281)

Chemical Formula: $C_{15}H_{10}FNO_3$

Molecular Weight: 271.25



Procedure: 1

Yield: 74%

State: white solid

^{1}H-NMR ($CDCl_3$): δ 4.07 (s, 3H), 7.14 (dd, J = 2.6, 8.2 Hz, 1H), 7.33 (td, J = 1.5, 8.6 Hz, 1H), 7.44 (t, J = 8.0 Hz, 1H), 7.49 (t, J = 8.0 Hz, 1H), 7.83 – 7.90 (m, 2H), 7.94 – 8.02 (m, 1H) ppm.

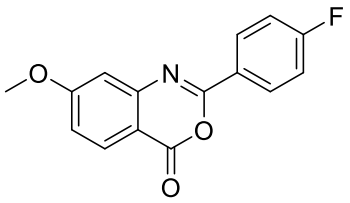
^{13}C-NMR ($CDCl_3$): δ 56.64 (CH_3, C-aromatic), 115.22 (CH, C-aromatic), 115.41 (CH, C-aromatic), 117.53 (CH, C-aromatic), 118.09 (C, C-aromatic), 119.64 (CH, C-aromatic), 124.09 (CH, C-aromatic), 129.03 (CH, C-aromatic), 130.35 (CH, C-aromatic), 132.62 (C, C-aromatic), 136.67 (C, C-aromatic), 154.50 (C, C-aromatic), 159.11 (C, C-aromatic), 161.88 (C, C-aromatic), 163.84 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$):δ -111.90 ppm.

Synthesis of 2-(4-fluorophenyl)-7-methoxy-4H-benzo[d][1,3]oxazin-4-one (282)

Chemical Formula: $C_{15}H_{10}FNO_3$

Molecular Weight: 271.25



Procedure: 1

Yield: 63%

State: white solid

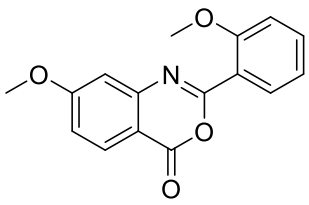
^{1}H-NMR ($CDCl_3$): δ 4.07 (s, 3H), 7.16 – 7.23 (m, 2H), 7.34 (dd, J = 1.1, 8.2 Hz, 1H), 7.49 (t, J = 8.0 Hz, 1H), 7.85 (dd, J = 1.2, 7.9 Hz, 1H), 8.34 – 8.43 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.60 (CH_3, C-aromatic), 116.03 (CH, C-aromatic), 117.42 (CH, C-aromatic), 117.87 (C, C-aromatic), 119.89 (CH, C-aromatic), 126.59 (C, C-aromatic), 128.65 (CH, C-aromatic), 130.87 (CH, C-aromatic), 136.93 (C, C-aromatic), 154.34 (C, C-aromatic), 159.29 (C, C-aromatic), 164.57 (C, C-aromatic), 166.59 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -106.02 ppm.

Synthesis of 7-methoxy-2-(2-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (283)

Chemical Formula: $C_{16}H_13NO_4$
Molecular Weight: 283.28



Procedure: 1

Yield: 82%

State: white solid

^{1}H-NMR ($CDCl_3$): δ 3.96 (s, 3H), 4.04 (s, 3H), 6.94 – 7.10 (m, 2H), 7.33 (d, J = 1.1 Hz, 1H), 7.46 – 7.454 (m, 2H), 7.86 (dd, J = 1.2, 7.9 Hz, 1H), 7.92 (dd, J = 1.7, 7.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.08 (CH_3, C-aromatic), 56.50 (CH_3, C-aromatic), 111.96 (CH, C-aromatic), 117.07 (CH, C-aromatic), 117.96 (C, C-aromatic), 119.57 (CH, C-aromatic), 120.52 (CH, C-aromatic), 120.72 (C, C-aromatic), 128.68 (CH, C-aromatic), 131.52 (CH, C-aromatic), 133.06 (CH, C-aromatic), 137.11 (C, C-aromatic), 154.39 (C, C-aromatic), 157.47 (C, C-aromatic), 158.79 (C, C-aromatic), 159.81 (C, C-aromatic) ppm.

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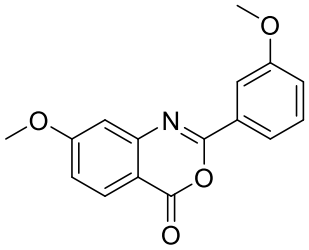
Synthesis of 7-methoxy-2-(3-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (284)

Chemical Formula: $C_{16}H_13NO_4$
Molecular Weight: 283.28



Procedure: 1

Yield: 85%

State: white solid

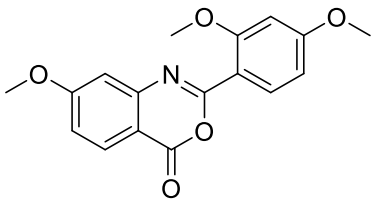
^{1}H-NMR ($CDCl_3$): δ 3.94 (s, 3H), 4.07 (s, 3H), 7.14 (dd, J = 2.6, 8.2 Hz, 1H), 7.32 – 7.38 (m, 1H), 7.44 (t, J = 8.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.83 – 7.90 (m, 2H), 7.95 – 8.00 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.58 (CH_3, C-aromatic), 56.60 (CH_3, C-aromatic), 112.73 (CH, C-aromatic), 117.40 (CH, C-aromatic), 118.05 (C, C-aromatic), 119.25 (CH, C-aromatic), 119.87 (CH, C-aromatic), 121.07 (CH, C-aromatic), 128.67 (CH, C-aromatic), 129.73 (CH, C-aromatic), 131.68 (C, C-aromatic), 136.90 (C, C-aromatic), 154.50 (C, C-aromatic), 156.39 (C, C-aromatic), 159.51 (C, C-aromatic), 159.86 (C, C-aromatic) ppm.

Synthesis of 2-(2,4-dimethoxyphenyl)-7-methoxy-4H-benzo[d][1,3]oxazin-4-one (285)

Chemical Formula: $C_{17}H_{15}NO_5$

Molecular Weight: 313.31



Procedure: 1

Yield: 56%

State: white solid

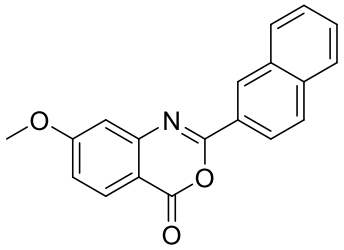
^{1}H-NMR ($CDCl_3$): δ 3.90 (s, 3H), 3.97 (s, 3H), 4.04 (s, 3H), 6.55 (d, J = 2.3 Hz, 1H), 6.60 (dd, J = 8.7, 2.3 Hz, 1H), 7.32 – 7.28 (m, 1H), 7.44 (t, J = 8.0 Hz, 1H), 7.83 (dd, J = 7.9, 1.2 Hz, 1H), 8.00 (d, J = 8.7 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.54 (CH_3, C-aromatic), 56.14 (CH_3, C-aromatic), 56.48 (CH_3, C-aromatic), 99.17 (CH, C-aromatic), 106.02 (CH, C-aromatic), 113.38 (C, C-aromatic), 116.93 (C, C-aromatic), 117.69 (CH, C-aromatic), 119.55 (CH, C-aromatic), 128.11 (CH, C-aromatic), 133.17 (CH, C-aromatic), 137.91 (C, C-aromatic), 154.23 (C, C-aromatic), 156.57 (C, C-aromatic), 159.98 (C, C-aromatic), 160.75 (C, C-aromatic), 164.00 (C, C-aromatic) ppm.

Synthesis of 7-methoxy-2(naphthalene-2-yl)-4H-benzo[d][1,3] oxazin-4-one (286)

Chemical Molecular Formula: $C_{19}H_{13}NO_3$

Molecular Weight: 303.1



Procedure: 1

Yield: 87%

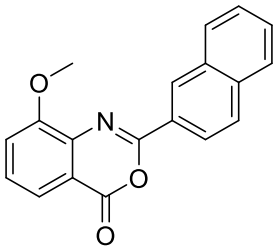
State: white powder

^{1}H-NMR ($CDCl_3$): δ 4.01 (s, 3H), 7.10 (dt, J = 4.6, 9.3 Hz, 1H), 7.54 – 7.65 (m, 1H), 7.68 – 7.73 (m, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.96 – 8.02 (m, 1H), 8.04 (dd, J = 8.0, 11.2 Hz, 1H), 8.19 – 8.26 (m, 1H), 8.39 (dd, J = 1.7, 8.7 Hz, 1H), 8.81 (s, 1H), 8.87 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.96 (CH_3, C-aliphatic), 108.96 (CH, C-aromatic), 109.86 (C, C-aromatic), 117.43 (CH, C-aromatic), 124.16 (CH, C-aromatic), 126.91 (CH, C-aromatic), 127.84 (CH, C-aromatic), 127.96 (C, C-aromatic), 128.36 (CH, C-aromatic), 128.59 (CH, C-aromatic), 129.41 (CH, C-aromatic), 129.62 (CH, C-aromatic), 130.33 (CH, C-aromatic), 132.85 (C, C-aromatic), 135.36 (C, C-aromatic), 149.57 (C, C-aromatic), 158.08 (C, C-aromatic), 159.39 (C, C-aromatic), 166.37 (C, C-aromatic) ppm.

Synthesis of 8-methoxy-(2-naphthalen-1-yl)-4H-benzo[d][1,3]oxazin-4-one (287)

Molecular Formula: $C_{19}H_{13}NO_3$
Molecular Weight: 303.31



Procedure: 1

Yield: 82%

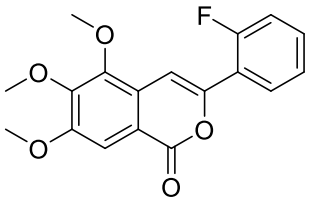
State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 4.09 (s, 3H), 7.38 (dd, J = 1.1, 8.2 Hz, 1H), 7.55 (t, J = 8.0 Hz, 1H), 7.57 – 7.63 (m, 2H), 7.68 – 7.76 (m, 1H), 7.87 – 7.96 (m, 2H), 8.06 (d, J = 8.2 Hz, 1H), 8.35 (dd, J = 1.2, 7.3 Hz, 1H), 9.20 (t, J = 9.8 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.59 (CH_3, C-aliphatic), 117.26 (CH, C-aromatic), 117.97 (C, C-aromatic), 119.62 (CH, C-aromatic), 124.76 (CH, C-aromatic), 125.70 (CH, C-aromatic), 126.34 (CH, C-aromatic), 127.28 (C, C-aromatic), 128.03 (CH, C-aromatic), 128.79 (CH, C-aromatic), 128.95 (CH, C-aromatic), 130.00 (CH, C-aromatic), 130.97 (C, C-aromatic), 133.06 (CH, C-aromatic), 135.54 (C, C-aromatic), 136.89 (C, C-aromatic), 154.65 (C, C-aromatic), 156.95 (C, C-aromatic), 159.76 (C, C-aromatic) ppm.

Synthesis of 2-(2-fluorophenyl)-6, 7, 8-trimethoxy-4H-benzo[d][1,3]oxazin-4-one (288)

Molecular Formula: $C_{17}H_{14}FNO_5$
Molecular Weight: 331.30



Procedure: 1

Yield: 79%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 4.007 (s, 3H), 4.093 (s, 3H), 4.208 (s, 3H), 7.22-7.26 (m, 1H), 7.30-7.32 (m, 1H), 7.48 (s, 1H), 7.54-7.56 (m, 1H), 8.15-8.18 (m, 1H) ppm.

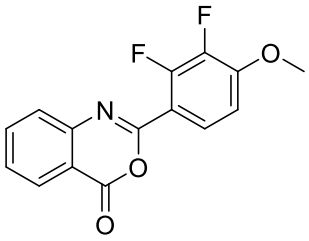
^{13}C-NMR ($CDCl_3$): δ 56.47 (CH_3, C-aliphatic), 61.55 (CH_3, C-aliphatic), 62.77 (CH_3, C-aliphatic), 103.89 (CH, C-aromatic), 112.39 (C, C-aromatic), 117.26 (CH, C-aromatic), 124.24 (CH, C-aromatic), 130.87 (CH, C-aromatic), 133.65 (CH, C-aromatic), 136.08 (C, C-aromatic), 148.06 (C, C-aromatic), 149.33 (C, C-aromatic), 152.28 (C, C-aromatic), 153.77 (C, C-aromatic), 159.25 (C, C-aromatic), 160.44 (C, C-aromatic), 162.51 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 112.22 ppm.

Synthesis of 2-(2, 3-difluoro-4-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (289)

Chemical Formula: $C_{15}H_9F_2NO_3$

Molecular Weight: 289.24



Procedure: 1

Yield: 94%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 3.93 (d, J = 2.3 Hz, 3H), 6.79 (dd, J = 6.0, 9.1 Hz, 1H), 7.44 – 7.52 (m, 1H), 7.62 (d, J = 7.7 Hz, 1H), 7.72 – 7.80 (m, 1H), 7.81 – 7.88 (m, 1H), 8.17 (dt, J = 5.6, 11.2 Hz, 1H) ppm.

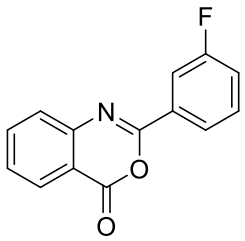
^{13}C-NMR ($CDCl_3$): δ 56.8 (CH_3, C-aliphatic), 107.82 (C, C-aromatic), 112.42 (CH, C-aromatic), 116.92 (C, C-aromatic), 125.20 (CH, C-aromatic), 125.24 (CH, C-aromatic), 127.36 (CH, C-aromatic), 128.59 (CH, C-aromatic), 136.66 (CH, C-aromatic), 140.91 (C, C-aromatic), 142.92 (C, C-aromatic), 146.79 (C, C-aromatic), 150.14 (C, C-aromatic), 152.17 (C, C-aromatic), 159.12 (C, C-aromatic)ppm.

^{19}F-NMR ($CDCl_3$): δ -133.10, -157.96 ppm.

Synthesis of 2 2-(3-fluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (290)

Chemical Formula: $C_{14}H_8FNO_2$

Molecular Weight: 241.22



Procedure: 1

Yield: 93%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 7.29 (t, J = 7.0 Hz, 1H), 7.45 – 7.62 (m, 2H), 7.71 (d, J = 7.9 Hz, 1H), 7.86 (t, J = 7.3 Hz, 1H), 7.99 (dd, J = 8.6, 20.7 Hz, 1H), 8.10 (d, J = 7.6 Hz, 1H), 8.25 (d, J = 7.6 Hz, 1H) ppm.

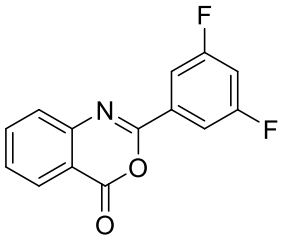
^{13}C-NMR ($CDCl_3$): δ 115.31 (CH, C-aromatic), 117.27 (CH, C-aromatic), 119.73 (CH, C-aromatic) 123.97 (CH, C-aromatic), 127.35 (CH, C-aromatic), 128.63 (CH, C-aromatic), 130.68 (CH, C-aromatic), 132.48 (C, C-aromatic), 136.69 (CH, C-aromatic), 146.64 (C, C-aromatic), 155.40 (C, C-aromatic), 159.17 (C, C-aromatic), 161.87 (C, C-aromatic), 163.84 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.54 ppm.

Synthesis of 2-(3, 5-difluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (291)

Chemical Formula: $C_{14}H_7F_2NO_2$

Molecular Weight: 259.21



Procedure: 1

Yield: 76%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 6.94 – 7.11 (m, 1H), 7.60 (td, J = 7.9, 1.1 Hz, 1H), 7.72 – 7.78 (m, 1H), 7.88 (dd, J = 5.9, 6.7 Hz, 3H), 8.28 (dd, J = 1.1, 7.9 Hz, 1H) ppm.

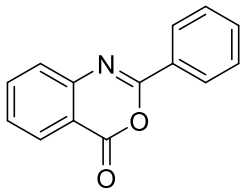
^{13}C-NMR ($CDCl_3$): δ 107.75 (C, C-aromatic), 108.15 (CH, C-aromatic), 111.21 (CH, C-aromatic), 111.43 (CH, C-aromatic), 117.13 (CH, C-aromatic), 127.49 (C, C-aromatic), 128.79 (CH, C-aromatic), 129.00 (CH, C-aromatic), 136.80 (CH, C-aromatic), 146.36 (C, C-aromatic), 155.15 (C, C-aromatic), 158.79 (C, C-aromatic), 162.39 (C, C-aromatic), 164.39 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -101.57, -109.89 ppm.

Synthesis of 2-phenyl-4H-benzo[d][1,3]oxazin-4-one (292)

Chemical Formula: $C_{14}H_9NO_2$

Molecular Weight: 223.23



Procedure: 1

Yield: 81%

State: white powder

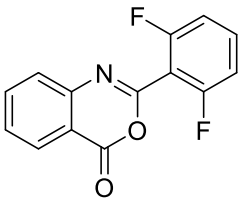
^{1}H-NMR ($CDCl_3$): δ 7.57 – 7.70 (m, 4H), 7.73 (dd, J = 0.5, 8.1 Hz, 1H), 7.92 – 7.99 (m, 1H), 8.14 – 8.24 (m, 3H) ppm.

^{13}C-NMR ($CDCl_3$): δ 116.93 (C, C-aromatic), 126.90 (CH, C-aromatic), 127.79 (CH, C-aromatic), 128.05 (CH, C-aromatic), 128.59 (CH, C-aromatic), 128.99 (CH, C-aromatic), 130.03 (C, C-aromatic), 132.71 (CH, C-aromatic), 136.85 (CH, C-aromatic), 146.25 (C, C-aromatic), 156.40 (C, C-aromatic), 158.85 (C, C-aromatic) ppm.

Synthesis of 2-(2, 6-difluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (293)

Chemical Formula: $C_{14}H_7F_2NO_2$

Molecular Weight: 259.21



Procedure: 1

Yield: 75%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 7.08 (t, J = 8.2 Hz, 2H), 7.47 – 7.57 (m, 1H), 7.65 (td, J = 1.1, 7.9 Hz, 1H), 7.76 (dd, J = 0.6, 8.1 Hz, 1H), 7.88 – 7.95 (m, 1H), 8.32 (dd, J = 1.1, 7.9 Hz, 1H) ppm.

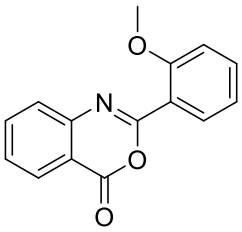
^{13}C-NMR ($CDCl_3$): δ 116.93 (C, C-aromatic), 127.13 (CH, C-aromatic), 128.17 (CH, C-aromatic), 129.84 (CH, C-aromatic), 134.25 (CH, C-aromatic), 134.33 (CH, C-aromatic), 134.42 (CH, C-aromatic), 137.14 (CH, C-aromatic), 145.41 (C, C-aromatic), 149.94 (C, C-aromatic), 158.36 (C, C-aromatic), 158.93 (C, C-aromatic), 160.96 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ - 110.48ppm.

Synthesis of 2-(2-methoxyphenyl)-4H-benzo[d][1,3]oxazin-4-one (294)

Chemical Formula: $C_{15}H_{11}NO_3$

Molecular Weight: 253.26



Procedure: 1

Yield: 66%

State: white powder

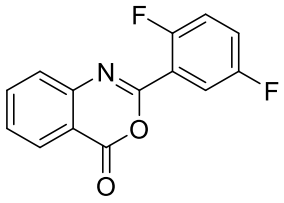
^{1}H-NMR ($CDCl_3$): δ 3.96 (s, 3H), 7.09 (dd, J = 9.9, 13.0 Hz, 2H), 7.48 – 7.60 (m, 2H), 7.73 (d, J = 8.0 Hz, 1H), 7.80 – 7.95 (m, 2H), 8.28 (dd, J = 1.3, 7.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.12 (CH_3, C-aliphatic), 112.17 (CH, C-aromatic), 116.98 (C, C-aromatic), 120.60 (CH, C-aromatic), 127.26 (CH, C-aromatic), 128.36 (CH, C-aromatic), 128.36 (CH, C-aromatic), 128.44 (CH, C-aromatic), 131.34 (CH, C-aromatic), 133.20 (CH, C-aromatic), 136.40 (C, C-aromatic), 147.06 (C, C-aromatic), 157.76 (C, C-aromatic), 158.64 (C, C-aromatic), 159.85 (C, C-aromatic) ppm

Synthesis of 2-(2, 5-difluorophenyl)-4H-benzo[d][1,3]oxazin-4-one (295)

Chemical Formula: $C_{14}H_7F_2NO_2$

Molecular Weight: 259.21



Procedure: 1

Yield: 94%

State: white powder

^{1}H-NMR ($CDCl_3$): δ 6.98 - 7.03 (m, 2H), 7.55 – 7.61 (m, 1H), 7.73 (d, J = 8.1 Hz, 1H), 7.84 – 7.91 (m, 1H), 8.20 (td, J = 6.4, 8.6 Hz, 1H), 8.28 (dd, J = 1.3, 7.9 Hz, 1H) ppm.

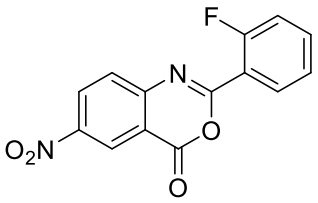
^{13}C-NMR ($CDCl_3$): δ 105.68 (CH, C-aromatic, d, J = 25 Hz), 112.08 (CH, C-aromatic, d, J = 20 Hz), 116.94 (C, C-aromatic), 127.41 (CH, C-aromatic), 128.66 (CH, C-aromatic), 128.85 (CH, C-aromatic), 132.74 (CH, C-aromatic), 136.74 (CH, C-aromatic), 141.01 (C, C-aromatic), 146.62 (C, C-aromatic), 159.07 (C, C-aromatic), 157.65 (C, C-aromatic), 158.77 (C, C-aromatic), 166.37 (C, C-aromatic) ppm

^{19}F-NMR ($CDCl_3$): δ - 102.25, - 103.46, ppm.

Synthesis of 2-(2-fluorophenyl)-6-nitro-4H-benzo[d][1,3]oxazin-4-one (296)

Chemical Formula: $C_{14}H_7FN_2O_4$

Molecular Weight: 286.22



Procedure: 1

Yield: 85%

State: yellow powder

^{1}H-NMR ($CDCl_3$): δ 7.45 – 7.55 (m, 1H), 7.67 (dd, J = 6.4, 14.3 Hz, 1H), 7.87 (d, J = 9.2 Hz, 1H), 7.92-7.98 (m, 1H), 8.58 (dd, J = 2.3, 8.7 Hz, 1H), 8.78 (t, J = 7.8 Hz, 1H), 8.88 – 8.92 (m, 1H) ppm.

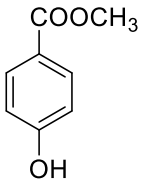
^{13}C-NMR ($CDCl_3$): δ 115.47 (CH, C-aromatic), 115.70 (C, C-aromatic), 119.31 (CH, C-aromatic), 124.26 (CH, C-aromatic), 126.50 (CH, C-aromatic), 127.73, 128.83 (CH, C-aromatic), 130.50 (CH, C-aromatic), 138.59 (CH, C-aromatic), 146.89 (C, C-aromatic), 150.21 (C, C-aromatic), 150.52 (C, C-aromatic), 152.64 (C, C-aromatic), 152.84 (C, C-aromatic), 159.12 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -114.81 ppm.

Synthesis of methyl 4-hydroxybenzoate (297)

Chemical Formula: $C_8H_8O_3$

Molecular Weight: 152.15



Procedure: 4- hydroxyl benxzoic acid (1 equivalent) was dissolved in methanol (3 mL/mmol) and hydrochloridric acid 12 N (100 µL/mmol) was added and the reaction mixture was heated at 85 °C for 24 hours. The product was evaporated and purified by silica gel colum chromatography.

Yield: 82%

State: white powder

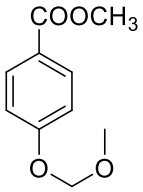
^{1}H-NMR ($CDCl_3$): δ 3.92 (s, 3H), 5.62 (bs, 1H), 7.03 – 6.86 (m, 2H), 8.18 – 7.84 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 52.40 (CH_3, C-aliphatic), 115.26 (CH, C-aromatic), 122.55 (C, C-aromatic), 131.96 (CH, C-aromatic), 160.03 (C, C-aromatic), 167.03 (C, C-aromatic) ppm.

Synthesis of methyl 4-(methoxymethoxy)benzoate (298)

Chemical Formula: $C_{10}H_{12}O_4$

Molecular Weight: 196.20



Procedure: To a solution of compound **297** (1 equivalent) and *N*,*N*-diisopropylethylamine (1.2 equivalent) in dichloromethane (2.5 mL/mmol) was added chloro methoxy methane (1.2 equivalent) at 0 °C and the reaction mixture was stirred at 25 °C for 16 hours. The reaction mixture was quenched with sodium carbonate, diluted with water, extracted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered and evaporated to give the desired product.

Yield: 84%

State: white powder

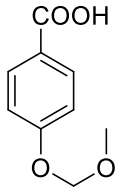
^{1}H-NMR ($CDCl_3$): δ 3.42 (s, 3H), 3.81 (s, 3H), 5.15 (s, 2H), 6.98 – 7.06 (d, J = 10 Hz, 2H), 7.96 – 8.03 (d, J = 9.8 Hz, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 51.47 (CH_3, C-aliphatic), 56.30 (CH_3, C-aliphatic), 94.10 (CH_2, C-aliphatic), 115.76 (CH, C-aromatic), 122.62 (C, C-aromatic), 132.27 (CH, C-aromatic), 161.63 (C, C-aromatic), 171.03 (C, C-aromatic) ppm.

Synthesis of 4-(methoxymethoxy)benzoic acid (299)

Chemical Formula: $C_9H_{10}O_4$

Molecular Weight: 182.18



Procedure: A solution of compound **298** (1 equivalent) in aqueous sodium hydroxide solution (1M, 1 mL/mmol) in 1,4-dioxane (1 mmol/1 mL) was stirred at 100 °C for 2 hours. HCl 1 M was added and the resulting white precipitate was collected under reduced pressure.

Yield: 78%

State: white powder

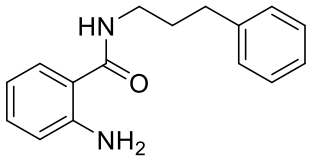
^{1}H-NMR ($CDCl_3$): δ 3.42 (s, 3H), 5.18 (s, 2H), 6.98 – 7.06 (m, 2H), 7.96 – 8.03 (m, 2H), 10.3 (bs, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 56.30 (CH_3, C-aliphatic), 94.10 (CH_2, C-aliphatic), 115.76 (CH, C-aromatic), 122.62 (C, C-aromatic), 132.27 (CH, C-aromatic), 161.63 (C, C-aromatic), 171.03 (C, C-aromatic) ppm.

Synthesis of 2-amino-N-(3-phenylpropyl)benzamide (300)

Chemical Formula: $C_{16}H_{18}N_2O$

Molecular Weight: 254.33



Procedure: To a stirred solution of anthranilic acid (1 equivalent) in dichloromethane (1 mL/ 1 mmol) was added 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.2 equivalents) and hydroxybenzotriazole (1.2 equivalents). The reaction mixture was stirred at room temperature for 30 minutes. To this, 3-phenylpropan-1-amine (1.5 equivalent) and the reaction mixture was stirred at room temperature for 16 hours. The reaction mixture was then, diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, filtered and evaporated to give the crude compound, which was then purified by silica gel chromatography.

Yield: 56 %

State: white powder

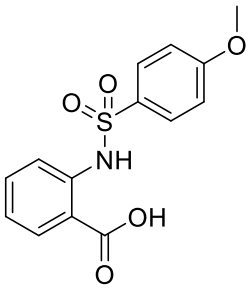
^{1}H-NMR ($CDCl_3$): δ 1.99 (dt, J = 7.3, 14.5 Hz, 2H), 2.76 (t, J = 7.6 Hz, 2H), 3.44 – 3.58 (m, 2H), 5.64 (s, 2H), 6.02 (s, 1H), 6.60 - 6.71 (m, 1H), 6.71 (dd, J = 0.9, 8.2 Hz, 1H), 7.16 (dd, J = 1.5, 7.9 Hz, 1H), 7.20 – 7.27 (m, 4H), 7.31 – 7.36 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 31.15 (CH_2, C-aliphatic), 33.55 (CH_2, C-aliphatic), 39.31 (CH_2, C-aliphatic), 116.06 (C, C-aromatic), 116.94 (CH, C-aromatic), 117.47 (CH, C-aromatic), 126.09 (CH, C-aromatic), 126.99 (CH, C-aromatic), 128.42 (CH, C-aromatic), 128.59 (CH, C-aromatic), 132.21 (CH, C-aromatic), 141.47 (C, C-aromatic), 148.21 (C, C-aromatic), 169.40 (C, C-aromatic) ppm.

Synthesis of 2-((4-methoxyphenyl)sulfonamido)benzoic acid (301)

Chemical Formula: $C_{14}H_{13}NO_5S$

Molecular Weight: 307.32



Procedure: 4

State: white powder

Yield: 55 %

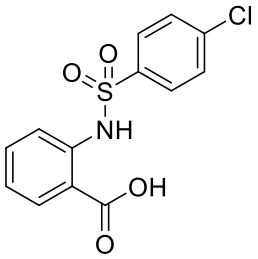
^{1}H-NMR ($CDCl_3$): δ 3.75 (s, 3H), 6.75 – 6.97 (m, 2H), 7.01 (dt, J = 5.6, 18.4 Hz, 1H), 7.18 (s, 1H), 7.44 (t, J = 8.5, 24.1 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 7.69 – 7.81 (m, 2H), 7.95 (d, J = 1.5 Hz, 1H), 10.21 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 55.61 (CH_3, C-aliphatic), 114.31 (CH, C-aromatic), 114.46 (C, C-aromatic), 118.91 (CH, C-aromatic), 122.95 (CH, C-aromatic), 129.50 (CH, C-aromatic), 130.79 (C, C-aromatic), 132.22 (CH, C-aromatic), 135.58 (CH, C-aromatic), 141.26 (C, C-aromatic), 163.29 (C, C-aromatic), 171.57 (C, C-aromatic) ppm.

Synthesis of 2-((4-chlorophenyl)sulfonamido)benzoic acid (302)

Chemical Formula: $C_{10}H_{13}ClNO_4S$

Molecular Weight: 311.74



Procedure: 4

State: white powder

Yield: 34 %

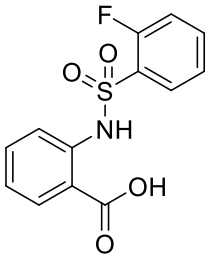
^{1}H-NMR ($CDCl_3$): δ 7.03 (dd, J = 17.6, 24.8 Hz, 1H), 7.20 (s, 1H), 7.36 (t, J = 12.0 Hz, 2H), 7.43 – 7.50 (m, 1H), 7.63 (d, J = 8.2 Hz, 1H), 7.74 (d, J = 8.7 Hz, 2H), 7.91 – 8.09 (m, 1H), 10.43 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 114.70 (C, C-aromatic), 118.99 (CH, C-aromatic), 123.45 (CH, C-aromatic), 128.47 (CH, C-aromatic), 128.72 (CH, C-aromatic), 129.48 (CH, C-aromatic), 130.06 (CH, C-aromatic), 132.34 (CH, C-aromatic), 135.67 (CH, C-aromatic), 137.75 (C, C-aromatic), 139.83 (C, C-aromatic), 140.72 (C, C-aromatic), 171.19 (C, C-aromatic) ppm.

Synthesis of 2-((2-fluorophenyl)sulfonamido)benzoic acid (303)

Chemical Formula: $C_{10}H_{13}FNO_4S$

Molecular Weight: 295.28



Procedure: 4

State: white powder

Yield: 63 %

^{1}H-NMR ($CDCl_3$): δ 7.10 (dt, J = 3.4, 14.0 Hz, 1H), 7.13 – 7.18 (m, 1H), 7.26 – 7.32 (m, 2H), 7.49 (ddd, J = 5.0, 10.8, 22.9 Hz, 1H), 7.61– 7.54 (m, 1H), 7.71 – 7.66 (m, 1H), 8.02 (td, J = 1.7, 7.8 Hz, 1H), 8.09 (dd, J = 1.5, 8.0 Hz, 1H), 10.86 (s, 1H) ppm.

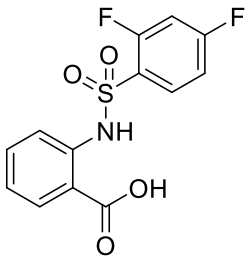
^{13}C-NMR ($CDCl_3$): δ 114.54 (C, C-aromatic), 117.20 (CH, C-aromatic), 117.37 (CH, C-aromatic, 123.00 (CH, C-aromatic), 124.37 (CH, C-aromatic, 124.40 (CH, C-aromatic), 130.98 (CH, C-aromatic), 132.32 (CH, C-aromatic), 135.65 (CH, C-aromatic), 140.49 (C, C-aromatic), 157.90 (C, C-aromatic), 159.94 (C, C-aromatic), 171.48 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -109.24 ppm.

Synthesis of 2-((2, 4-difluorophenyl)sulfonamido)benzoic acid (304)

Chemical Formula: $C_{13}H_9F_2NO_4S$

Molecular Weight: 313.27



Procedure: 4

State: white powder

Yield: 65 %

^{1}H-NMR ($CDCl_3$): δ 6.91 (t, J = 8.1 Hz, 1H), 7.01 (t, J = 8.1 Hz, 1H), 7.13 (t, J = 7.6 Hz, 1H), 7.52 (t, J = 7.8 Hz, 1H), 7.67 (d, J = 8.4 Hz, 1H), 7.97 – 8.06 (m, 1H), 8.10 (d, J = 7.8 Hz, 1H), 10.91 (s, 1H) ppm.

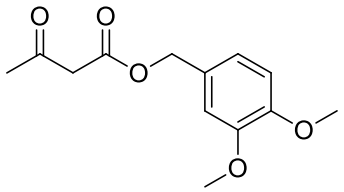
^{13}C-NMR ($CDCl_3$): δ 114.54 (C, C-aromatic), 117.20 (CH, C-aromatic), 117.37 (CH, C-aromatic, 123.00 (CH, C-aromatic), 124.37 (CH, C-aromatic, 124.40 (CH, C-aromatic), 130.98 (CH, C-aromatic), 132.32 (CH, C-aromatic), 135.65 (CH, C-aromatic), 140.49 (C, C-aromatic), 157.90 (C, C-aromatic), 159.94 (C, C-aromatic), 171.48 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 99.04, - 103.65 ppm.

Synthesis of 3,4-dimethoxybenzyl 3-oxobutanoate (305)

Chemical Formula: $C_{13}H_{16}O_5$

Molecular Weight: 252.26



Procedure: A homogenous mixture of 3, 4-dimethoxybenzylalchol (1.4 equivalent) and methyl aceto-acetate (1 equivalent) was heated at 110 °C for 24 hours. The mixture was purified by silica gel column using ethyl acetate/*n*-hexane (1:2) as eluent.

State: yellow oil

Yield: 48 %

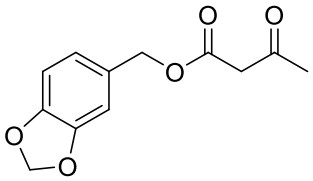
^{1}H-NMR ($CDCl_3$): δ 2.28 (d, *J* = 14.9 Hz, 3H), 3.49 (s, 1H), 3.51 (s, 1H), 3.76 (s, 3H), 3.90 (s, 3H), 4.65 (s, 1H), 5.14 (s, 1H), 6.86 (d, *J* = 1.8, 8.0 Hz, 1H), 6.91 (dd, *J* = 1.8, 6.4Hz, 1H), 6.95 (dd, *J* = 1.8, 6.9 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.15 (CH_3, C-aliphatic), 30.89 (CH_3, C-aliphatic), 50.12 (CH_2, C-aliphatic), 55.93 (CH_3, C-aliphatic), 67.23 (CH_2, C-aliphatic), 111.07 (CH, C-aromatic), 111.89 (CH, C-aromatic), 121.39 (CH, C-aromatic), 127.83 (C, C-aromatic), 149.09 (C, C-aromatic), 149.32 (C, C-aromatic), 166.98 (C, C-aromatic), 200.26 (C, C-aromatic) ppm.

Synthesis of benzo[d][1,3]dioxol-5-ylmethyl 3-oxobutanoate (306)

Chemical Formula: $C_{12}H_{12}O_5$

Molecular Weight: 236.22



Procedure: 2, 2, 6- dimethyl-4H-1,3-dioxin-4-one (1.3 equivalent) and alcohol (1 equivalent) was mixed with potassium acetate (4 equivalent) and the mixture was heated at 130 °C for 6 hours. The crude compound was then purified by automated flash column chromatography with the dichloromethane/methanol eluent (9:1).

State: brown oil

Yield: 90 %

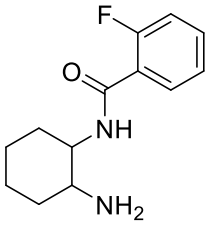
^{1}H-NMR ($CDCl_3$): δ 2.26 (s, 2H), 3.47 (s, 3H), 5.03 (s, 2H), 5.90 – 5.93 (m, 2H), 6.76 (dd, *J* = 6.5, 9.9 Hz, 1H), 6.82 (dd, *J* = 8.4, 10.0 Hz, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 30.30 (CH_3, C-aliphatic), 50.13 (CH_2, C-aliphatic), 66.84 (CH_2, C-aliphatic), 101.21 (C, C-aromatic), 108.83 (CH, C-aromatic), 109.09 (CH, C-aromatic), 122.46 (CH, C-aromatic), 128.97 (C, C-aromatic), 147.91 (C, C-aromatic), 166.65 (C, C-aromatic), 200.12 (C, C-aromatic) ppm.

Synthesis of *N*-(2-aminocyclohexyl)-2-fluorobenzamide (307)

Chemical Formula: $C_{13}H_{17}FN_2O$

Molecular Weight: 236.29



Procedure: 9-BBN (1 equivalent) was added to a solution of (R, R) - (-) – 1, 2-diaminocyclohexane (1 equivalent) in dry THF (5 mL/1 mmol) and the reaction mixture was stirred at 25 °C for 1 hour. Benzoyl chloride (1 equivalent) was then added and the reaction mixture was stirred for a further 2 hours at 25 °C. Reaction mixture was quenched with water, extracted with ethyl acetate, washed with sodium hydroxide aqueous solution 1M, dried over $MgSO_4$ filtered and evaporated to give a yellow oil, which was then purified by automated flash column chromatography using a mixture of dichloromethane, methanol and 0.1% of TEA to give the titled compound as a white powder.

State: pale yellow powder

Yield: 56%

^{1}H-NMR ($CDCl_3$): δ 1.11 – 1.25 (m, 2H), 1.30 – 1.39 (m, 1H), 1.5 (s, 2H, NH_2), 1.60 (s, 2H), 1.60 – 1. 73 (m, 1H), 1.90 – 1.99 (m, 1H), 2.03 (dd, J = 3.9, 10.9 Hz, 1H), 2.44 (td, J = 4.0, 10.3 Hz, 1H), 3.67 – 3.78 (m, 1H), 6.53 (s, 1H, NH), 6.95 – 7.08 (m, 1H), 7.17 – 7.23 (m, 1H), 7.40 (dd, J = 5.3, 9.2 Hz, 1H), 8.01 (tt, J = 1.7, 7.9 Hz, 1H) ppm.

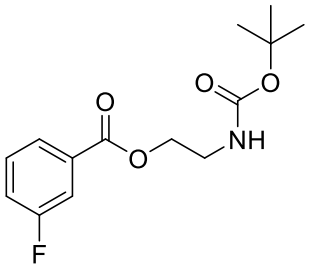
^{13}C-NMR ($CDCl_3$): δ 25.11 (CH_2, C-aliphatic), 32.49 (CH_2, C-aliphatic), 35.40 (CH_2, C-aliphatic), 55.67 (CH_3, C-aliphatic), 56.79 (CH_3, C-aliphatic), 115.92 (CH, C-aromatic), 116.12 (C, C-aromatic), 121.00 (CH, C-aromatic), 124.85 (CH, C-aromatic), 133.23 (CH, C-aromatic), 141.58 (C, C-aromatic), 159.48 (C, C-aromatic), 161.79 (C, C-aromatic), 163.43 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ – 113.63 ppm.

Synthesis of 2-((tert-butoxycarbonyl)amino)ethyl 3-fluorobenzoate(308)

Chemical Formula: $C_{14}H_{18}FNO_4$

Molecular Weight: 283.30



Procedure: 9

State: white powder

Yield: 76 %

^{1}H-NMR ($CDCl_3$): δ 1.47 (s, 9H), 3.46 (d, J = 5.1 Hz, 2H), 4.26 (t, J = 5.3 Hz, 2H), 7.02 – 7.11 (bs, 1H, NH), 7.38 – 7.52 (m, 2H), 7.75 (dd, J = 2.5, 9.3 Hz, 1H), 7.84 – 7.96 (m, 1H) ppm.

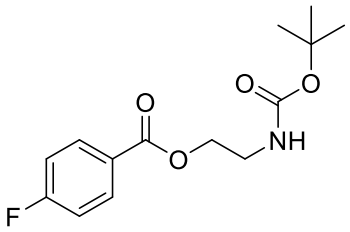
^{13}C-NMR ($CDCl_3$): δ 28.35 (CH_3, C-aliphatic), 39.71 (CH_2, C-aromatic), 64.65 (CH_2, C-aromatic), 116.51 (CH, C-aromatic), 120.14 (CH, C-aromatic), 125.46 (CH, C-aromatic), 130.10 (CH, C-aromatic), 132.28 (C, C-aromatic), 158.83 (C, C-aromatic), 161.42 (C, C-aromatic), 163.43 (C, C-aromatic), 165.42 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -111.98 ppm.

Synthesis of 2-((tert-butoxycarbonyl)amino)ethyl 4-fluorobenzoate (309)

Chemical Formula: $C_{14}H_{18}FNO_4$

Molecular Weight: 283.30



Procedure: 9

State: white powder

Yield: 76 %

^{1}H-NMR ($CDCl_3$): δ 1.37 (s, 9H), 3.45 (t, J = 12.6 Hz, 2H), 4.36 (t, J = 5.3 Hz, 2H), 7.1 (s, 1H, NH), 6.90 – 7.00 (m, 2H), 7.92 – 8.06 (m, 2H) ppm.

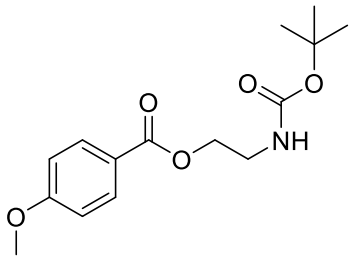
^{13}C-NMR ($CDCl_3$): δ 28.35 (CH_3, C-aliphatic), 39.76 (CH_2, C-aliphatic), 64.38 (CH_2, C-aliphatic), 115.47 (C, C-aromatic), 115.51 (CH, C-aromatic), 126.15 (C, C-aromatic), 132.22 (CH, C-aromatic), 164.88 (C, C-aromatic) 165.57 (C, C-aromatic), 166.90 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -105.30 ppm.

Synthesis of 2-((tert-butoxycarbonyl)amino)ethyl 4-fluorobenzoate (310)

Chemical Formula: $C_{15}H_{21}NO_5$

Molecular Weight: 295.34



Procedure: 9

State: white powder

Yield: 65 %

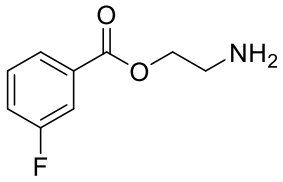
^{1}H-NMR ($CDCl_3$): δ 1.46 (s, 9H), 3.55 (s, 2H), 3.85 (s, 3H), 4.39 (t, J = 5.2 Hz, 2H), 7.01 (bs, 1H, NH), 7.14 (d, J = 8.6 Hz, 2H), 8.09 (d, J = 9.0 Hz, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 28.38 (CH_3, C-aliphatic), 29.73 (CH_2, C-aliphatic), 39.87 (CH_3, C-aliphatic), 64.01 (CH_2, C-aliphatic), 113.65 (CH, C-aromatic), 122.27 (C, C-aromatic), 131.75 (CH, C-aromatic), 155.84 (C, C-aromatic), 163.51 (C, C-aromatic), 166.30 (C, C-aromatic) ppm.

Synthesis of 2-aminoethyl 3-fluorobenzoate (311)

Chemical Formula: $C_9H_{10}FNO_2$

Molecular Weight: 183.18



Procedure: 10

State: orange powder

Yield: 48 %

^{1}H-NMR ($CDCl_3$): δ 2.40 (s, 2H), 3.66 (dd, J = 5.4, 10.2 Hz, 2H), 3.82 – 3.91 (m, 2H), 6.71 (s, 1H), 7.22 (td, J = 2.5, 8.3 Hz, 1H), 7.42 (tt, J = 7.0, 14.1 Hz, 1H), 7.48 – 7.59 (m, 1H) ppm.

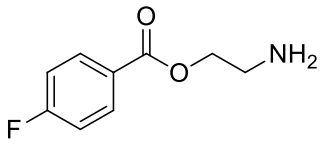
^{13}C-NMR ($CDCl_3$): δ 42.79 (CH_2, C-aliphatic), 61.12 (CH_2, C-aliphatic), 114.53 (CH, C-aromatic), 115.71 (CH, C-aromatic), 124.84 (C, C-aromatic), 132.47 (CH, C-aromatic), 132.73 (CH, C-aromatic), 161.73 (C, C-aromatic), 165.18 (C, C-aromatic) ppm.

^{19}F-NMR ($CDCl_3$): δ -112.18 ppm.

Synthesis of 2-aminoethyl 4-fluorobenzoate (312)

Chemical Formula: $C_9H_{10}FNO_2$

Molecular Weight: 183,18



Procedure: 10

State: yellow powder

Yield: 33%

^{1}H-NMR ($CDCl_3$): δ 1.43 (bs, 2H), 3.24 (s, 2H), 4.44 (s, 2H), 6.95 (d, J = 8.4 Hz, 2H), 7.97 (dd, J = 5.4, 8.5 Hz, 2H) ppm.

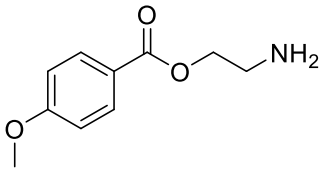
^{13}C-NMR ($CDCl_3$): δ 39.96 (CH_2, C-aliphatic), 61.22 (CH_2, C-aliphatic), 115.71 (CH, C-aromatic), 124.72 (C, C-aromatic), 132.48 (CH, C-aromatic), 132.55 (C, C-aromatic), 166.26 (C, C-aromatic) ppm.

^{19}F-NMR (CDCl3): δ -103.59 ppm.

Synthesis of 2-aminoethyl 4-methoxybenzoate (313)

Chemical Formula: $C_{10}H_{13}NO_3$

Molecular Weight: 195.22



Procedure: 10

State: colourless oil

Yield: 71 %

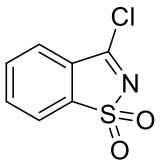
^{1}H-NMR ($CDCl_3$): δ 1.44 (bs, 2H), 3.36 (s, 2H), 3.71 – 3.82 (m, 2H), 4.28 (s, 3H), 6.83 (dd, J = 2.6, 8.9 Hz, 2H), 7.77 – 7.94 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 42.90 (CH_2, C-aliphatic), 55.42 (CH_3, C-aliphatic), 62.50 (CH_2, C-aliphatic), 113.78 (CH, C-aromatic), 126.36 (C, C-aromatic), 128.84 (CH, C-aromatic), 162.33 (C, C-aromatic), 168.24 (C, C-aromatic) ppm.

Synthesis of 3-chlorobenzo[d]wasothiazole 1,1-dioxide (314)

Chemical Formula: $C_7H_4ClNO_2S$

Molecular Weight: 201.63



Procedure: To a solution of 2, 3-dihydroxy-1,1-benzothiazol-3-one-1,1-dioxide (1 equivalent) in dioxane (1mmol/1mL), *N*,*N*-dimethylformamide (0.1 equivalent) and thionyl chloride (1.5 equivalent) was added and the reaction mixture was stirred at 110 °C for 48 hours. The thionyl chloride was evaporated and the crude product was used without further purification.

State: pale yellow slurry

Yield: 70%

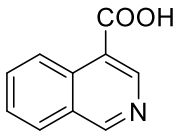
^{1}H-NMR ($CDCl_3$): δ 7.82 – 7.94 (m, 2H), 7.97 (d, *J* = 7.3 Hz, 1H), 8.07 (d, *J* = 7.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 122.47 (CH, C-aromatic), 125.09 (CH, C-aromatic), 129.92 (C, C-aromatic), 134.43(CH, C-aromatic), 135.12 (CH, C-aromatic), 140.62 (C, C-aromatic), 166.08 (C, C-aromatic) ppm.

Synthesis of isoquinoline-4-carboxylic acid (315)

Chemical Formula: $C_{10}H_7NO_2$

Molecular Weight: 173.17



Procedure: 4-bromoisoquinoline (1 equivalent) in anhydrous tetrahydrofuran (1 ml/ mmol) was cooled at 0 °C under N_2 and then a solution of t-BuLi (2 equivalent) was added drop-wise via syringe and stirred at -80 °C for 20 minutes. The reaction mixture was poured into dry ice and quenched with water and an aqueous solution of sodium hydroxide 1M. The reaction mixture was extracted with ethyl acetate, acidified with hydrochloric acid 12N and the desired product was collected as a precipitate.

State: white powder

Yield: 68 %

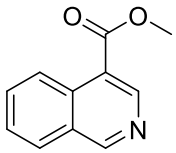
^{1}H-NMR ($CDCl_3$): δ 7.19 (s, 2H), 7.65 – 7.76 (m, 1H), 7.90 – 8.03 (m, 1H), 8.41 (d, J = 8.4 Hz, 1H), 8.99 – 9.16 (m, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 125.77 (CH, C-aromatic), 126.75 (CH, C-aromatic), 127.10 (C, C-aromatic), 129.04 (CH, C-aromatic), 133.16 (CH, C-aromatic), 135.91 (C, C-aromatic), 146.44 (CH, C-aromatic), 157.47 (C, C-aromatic), 169.02 (C, C-aromatic) ppm.

Synthesis of methyl isoquinoline-4-carboxylate (316)

Chemical Formula: $C_{11}H_9NO_2$

Molecular Weight: 187.20



Procedure: To a stirred solution of compound **315** (1 equivalent) in anhydrous dichloromethane (1 ml/ mmol) was added EDCI (1.2 equivalent) and HOBt (1.2 equivalent). The reaction mixture was stirred 20 minutes at 25 °C. Methanol (1.5 equivalent) was added and the reaction mixture was stirred at 25 °C for 16 hours. The reaction mixture was diluted with water, extracted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered and evaporated to give the final product as a white powder, purified by automated flash column chromatography using chloroform/methanol as eluent.

State: white powder

Yield: 72 %

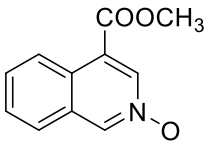
^{1}H-NMR ($CDCl_3$): δ 3.96 (s, 3H), 7.60 (dd, J = 6.9, 8.1 Hz, 1H), 7.77 (dd, J = 6.9, 8.5 Hz, 1H), 7.96 (d, J = 8.2 Hz, 1H), 8.87 (dd, J = 0.8, 8.7 Hz, 1H), 9.11 (s, 1H), 9.30 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 52.33 (CH_3, C-aliphatic), 120.51 (C, C-aromatic), 125.08 (CH, C-aromatic), 127.70 (CH, C-aromatic), 128.28 (CH, C-aromatic), 128.51 (C, C-aromatic), 132.28 (CH, C-aromatic), 133.88 (C, C-aromatic), 146.81 (CH, C-aromatic), 156.97 (CH, C-aromatic), 166.91 (C, C-aromatic) ppm.

Synthesis of methyl 2-(l1-oxidaneyl)-2l4-isoquinoline-4-carboxylate (317)

Chemical Formula: $C_{11}H_9NO_3$

Molecular Weight: 203.20



Procedure: To a stirred solution of compound **316** (1 equivalent) in anhydrous dichloromethane (2.5 ml/ mmol) was added meta-chloroperoxybenzoic acid (1.2 equivalent) portion wise at 0 °C. The reaction mixture was stirred overnight while allowing it to attain at 25 °C for 14 hour.

The reaction mixture was diluted with water, extracted with dichloromethane, washed with sodium bicarbonate, dried over $MgSO_4$, filtered and evaporated to give a white powder purified by automated flash column chromatography.

State: white powder

Yield: 84 %

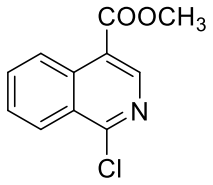
^{1}H-NMR ($CDCl_3$): δ 3.95 (s, 3H), 7.53 – 7.65 (m, 2H), 7.67 – 7.70 (m, 1H), 8.69 (s, 1H), 8.75 (d, J = 8.2 Hz, 1H), 8.80 (s, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 53.04 (CH_3, C-aliphatic), 125.07 (C, C-aromatic), 125.44 (CH, C-aromatic), 125.55 (CH, C-aromatic), 126.95 (C, C-aromatic), 129.94 (CH, C-aromatic), 130.17 (C, C-aromatic), 130.65 (CH, C-aromatic), 139.41 (CH, C-aromatic), 140.22 (CH, C-aromatic), 164.15 (C, C-aromatic) ppm.

Synthesis of methyl 1-chloroisoquinoline-4-carboxylate (318)

Chemical Formula: $C_{11}H_8ClNO_2$

Molecular Weight: 221.64



Procedure: Compound **317** (1 equivalent) in anhydrous dichloromethane (1 ml/ mmol) was stirred under N_2 and $POCl_3$ (3 equivalent) was added. DMF in catalytic amount was added. The reaction mixture was heated at 160 °C for 8 hours. The reaction mixture was quenched with sodium carbonate, diluted with water, extracted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered and evaporated to give a black oil, purified by automated flash column chromatography.

State: white powder

Yield: 85 %

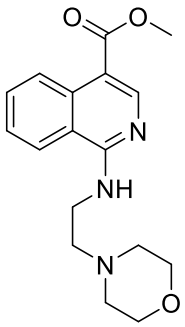
^{1}H-NMR ($CDCl_3$): δ 3.86 (d, J = 8.8 Hz, 3H), 7.49 (dt, J = 7.7, 26.7 Hz, 1H), 7.59 – 7.69 (m, 1H), 8.15 (d, J = 8.4 Hz, 1H), 8.66 – 8.80 (m, 2H) ppm.

^{13}C-NMR ($CDCl_3$): δ 52.51 (CH_3, C-aliphatic), 120.69 (C, C-aromatic), 125.71 (CH, C-aromatic), 126.93 (CH, C-aromatic), 128.88 (CH, C-aromatic), 132.84 (CH, C-aromatic), 135.61 (C, C-aromatic), 145.41 (CH, C-aromatic), 156.51 (C, C-aromatic), 160.75 (C, C-aromatic), 166.41 (C, C-aromatic) ppm.

Synthesis of methyl 1-((2-morpholinoethyl)amino)isoquinoline-4-carboxylate (319)

Chemical Formula: $C_{17}H_{21}N_3O_3$

Molecular Weight: 315.37



Procedure: In a microwave vial, to a stirred solution of compound **318** (1 equivalent) in anhydrous pyridine (3 ml/mmol) was added 2-morpholinoethan-1-amine (1.22 equivalent). The reaction mixture was heated at 100 °C, 80 W, 10 minutes. The reaction mixture was then diluted with water, extracted with dichloromethane, washed with brine, dried over $MgSO_4$, filtered and evaporated to give a yellow oil, which was purified by automated flash column chromatography (chloroform/methanol, 9:1) as a yellow oil.

State: yellow oil

Yield: 70 %

^{1}H-NMR ($CDCl_3$): δ 2.46 (s, 4H), 2.58 – 2.69 (m, 2H), 3.58 -3.68 (m, 6H), 3.84 (d, J = 5.2 Hz, 3H), 6.55 (s, 1H), 7.35 – 7.47 (m, 1H), 7.58 – 7.63 (m, 1H), 7.65 – 7.76 (m, 1H), 8.74 (d, J = 5.8 Hz, 1H), 8.93 (d, J = 8.6 Hz, 1H) ppm.

^{13}C-NMR ($CDCl_3$): δ 37.54 (CH_2, C-aliphatic), 51.46 (CH_3, C-aliphatic), 53.18 (CH_2, C-aliphatic), 56.58 (CH_2, C-aliphatic), 67.06 (CH_2, C-aliphatic), 109.49 (C, C-aromatic), 117.11 (C, C-aromatic), 121.60 (CH, C-aromatic), 125.91 (CH, C-aromatic), 126.12 (CH, C-aromatic), 130.96 (CH, C-aromatic), 135.11 (C, C-aromatic), 148.93 (CH, C-aromatic), 157.76 (C, C-aromatic), 167.56 (C, C-aromatic) ppm.