A new series of bicalutamide, enzalutamide and enobosarm derivatives carrying pentafluorosulfanyl (SF_5) and pentafluoroethyl (C_2F_5) substituents: improved antiproliferative agents against prostate cancer.

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Abstract: SAR studies on bicalutamide, enobosarm and enzalutamide analogues, functionalised with polyfluorinated groups, is presented. Among the novel bicalutamide and enobosarm derivatives synthesised, several displayed significantly improved *in vitro* anticancer activity, with IC_{50} values in the low micromolar range against four different prostate cancer cell lines (LNCaP, VCaP, DU-145 and 22Rv1), showing up to 48-fold increase in comparison with the parent structures. In particular, SF_5 enobosarm analogues were found to be most potent compounds, full AR antagonists and with favourable ADME properties. The most promising compound (**48a**) was evaluated for its *in vivo* efficacy in PC xenograft mouse model (22Rv1) with results comparable to the standard-of-care docetaxel.

Introduction

In the recent years, incorporation of fluorine and fluorine-containing groups into organic molecules to perturb the chemical, physical and biological properties of the parent compounds has proved to be a very successful strategy in drug design.[1-6] Fluorine atom in organic molecules, can impart a unique set of properties due to the distinctive combination of electronegativity, size and lipophilicity.[7-9] These factors combined have a substantial impact on molecular conformation of the drug, which may affect the binding affinity to the target protein. For these reasons, organo-fluorine structures have received and they are still receiving increasing attention in medicinal

chemistry,[10-15] and currently, fluorine is found in about 20% of pharmaceuticals.[13] The trifluoromethyl,[16-18] difluoromethyl[19-22] and fluoromethyl groups[23] (in decreasing order)[24] are the most exploited fluorinated groups in medicinal chemistry. In contrast, polyfluoroalkyl groups have been little exploited in medicinal chemistry compared to the above groups.[25, 26] However, recent years have witnessed an increased use of higher polyfluorinated groups in drug candidate molecules. Examples of these are the pentafluoroethyl (C_2F_5) and the pentafluorosulfanyl groups (SF_5).[27] The C_2F_5 group shows a broad synthetic access, whereas incorporation of SF_5, group is more challenging. The intrinsic difficulty of its introduction into a variety of substrates due to the harsh conditions needed has often precluded the investigation of its use in medicinal chemistry. Nevertheless, the last few years have witnessed an increased use of this functional group, especially in material chemistry, due to the appearance of novel and safe synthetic methodologies.[28, 29] Nowadays more and more building blocks functionalised with the SF_5 group are commercially available making the pentafluorosulfanyl group increasingly used even among medicinal chemists.[30-34] A few reports on drugs functionalised with SF_5 and C_2F_5 have showed an improvement in their physiological and / or biological properties compared to the parent drugs.[34-40] The breast cancer therapeutic agent fulvestrant (Faslodex®, Figure.1), is the only approved drug containing a C_2F_5 group. Studies on its mode of action have shown that its unique C-7 side chain with a terminal C_2F_5 group is responsible for the increased metabolic stability during the receptor binding.[41] In 2017, the first clinical candidate containing a SF_5 group (DSM265) reached Phase IIa trials for the treatment of malaria (Figure.1).[42] During these studies, an aniline ring with a 4-SF_5 motif provided the optimal balance between parasite activity, metabolic stability, and good pharmacokinetic and safety profiles in patients.[43]

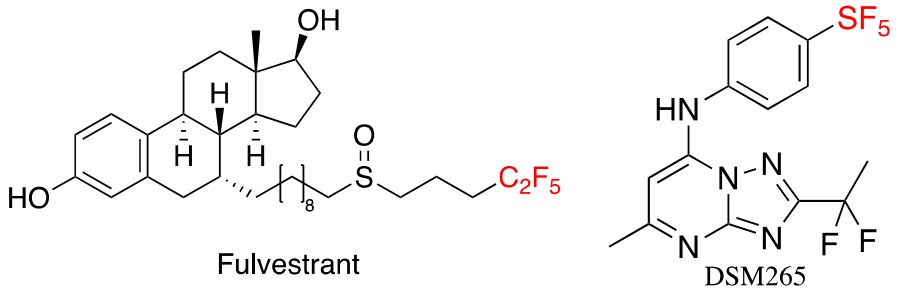


Figure 1: Structure of the anticancer drug Fulvestrant and the antimalarial agent DSM265.

Introduction of fluorine and polyfluorinated units such as the trifluoromethyl group has been a successful strategy in the development of efficacious drugs for the treatment of Prostate Cancer

(PC) (Figure 2). PC is one of the major causes of death of men in worldwide.[44] The initial treatment of PC is often androgen deprivation therapy (ADT). Unfortunately, after an initial response to ADT, the vast majority of patients will go on to develop the most aggressive form of this disease called castration resistant (CRPC) disease within a median timespan of 2-3 years.[45, 46] CRPC still depends on androgen stimulation for its growth and as such the use of androgen receptor (AR) inhibitors is still useful either alone or in combination with other therapies.[47]

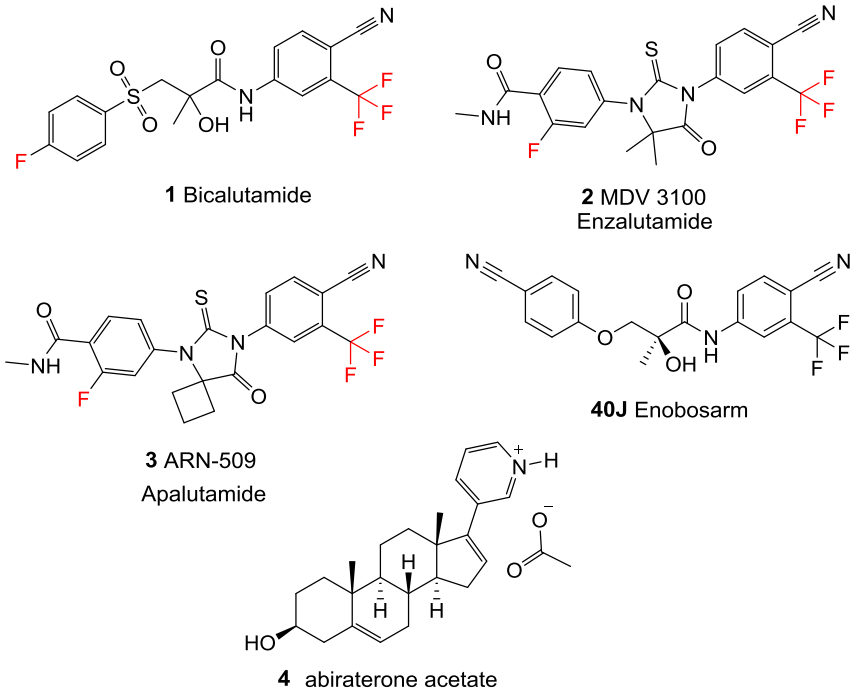


Figure 2: Structures of non-steroidal anti-androgens (**1-3**) and abiraterone acetate (**4**), drugs approved for the treatment of various stages of Prostate Cancer.

One of the problems associated with the use of bicalutamide, and similar compounds is that their prolonged use not only causes resistance but theyir use enhance the tumor growth. Efforts to contrast this resistance resulted in the development of novel and high affinity anti-androgens, which extended the median survival time of CRPC patients. Molecules such as enzalutamide (Xtandi®, MDV3100, **2**) and the most recently approved apalutamide (ARN-509, **3**, for non-metastatic PC) and abiraterone acetate (Zytiga® **4**) are now extensively used in PC therapy. Unfortunately, despite a favorable profile of these drugs, patients with CPRC and those who are initially sensitive eventually begin to develop secondary resistance also to these drugs as for example reported for enzalutamide.[50] Despite the resistance problem, ASCO recommends “men

with metastatic CRPC to continue hormone therapy to keep androgen levels low regardless of the other treatments used".[51] In this scenario it is evident that the search for more potent agent against prostate cancer is still imperative. In our recent communications[52, 53] we have demonstrated that the introduction of fluorinated groups into selected positions of the aromatic rings of the bicalutamide (**1)** and enzalutamide (**2)** allowed us to obtain new compounds with improved *in vitro* anti-proliferative activity in the most common PC cell lines. We were the first to demonstrate that depending on the position of these fluorinated groups we could convert the partially AR agonist enobosarm into a full AR antagonist molecule with the potential to become a PC drug.[53] Moreover, structure–activity relationship (SAR) studies on bicalutamide-like molecules have shown that electron-poor ring A, (e.g bearing CN, NO_2 or CF_3) is required for a strong ligand–AR binding.[54] These findings prompted us to explore the effects of the polyfluorinated groups SF_5 and C_2F_5 on the antiproliferative activity of bicalutamide, enobosarm and enzalutamide in PC cell lines.

The SF_5 group, considered as a CF_3, *tert*-butyl, halogen, or nitro groups bioisoster,[55] is stable under physiological conditions[56] and possess unique physical and chemical properties such as a great electronegativity coupled with an unusual lipophilicity, which might have an impact in the activity considering the very hydrophobic environment of the AR binding pocket). SF_5 has also a different electron density profile (pyramidal for SF_5 opposite to spherical for CF_3) as well as a larger molar volume than CF_3 that may be beneficial for biological activity.[55] On other hand the C_2F_5 group is reported to offer similar properties to SF_5 such as comparable lipophilicity, while slightly less electronegative. It shows also high chemical and metabolic stability with a size intermediate between CF_3 and *tert*-butyl. Therefore, selected enobosarm candidates, were also functionalised with the bulkier, but electronically similar to CF_3, C_2F_5 group, and evaluated for their antiproliferative activity.

In this article, we would like to report our findings on the synthesis and the biological evaluation including antiproliferative activity on four PC cell lines, of SF_5 and C_2F_5 substituted bicalutamide/enobosarm and enzalutamide analogues (Figure 3). For selected structures the AR antagonist/agonist behaviour as well as the *in vitro* ADME properties are reported. The lead compound was evaluated for its ability to impair tumor growth in PC xenograft mouse model.

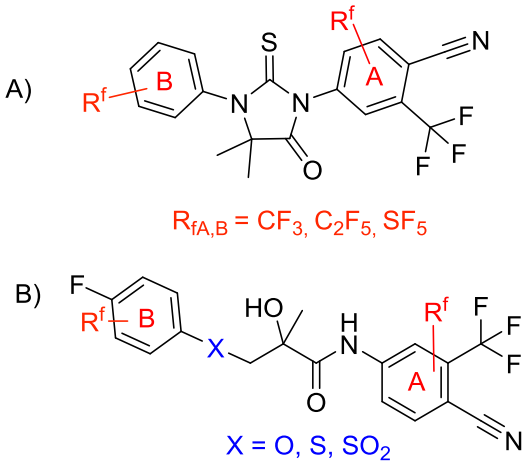


Figure 3. General structures of the compounds investigated in this study

Results and discussion

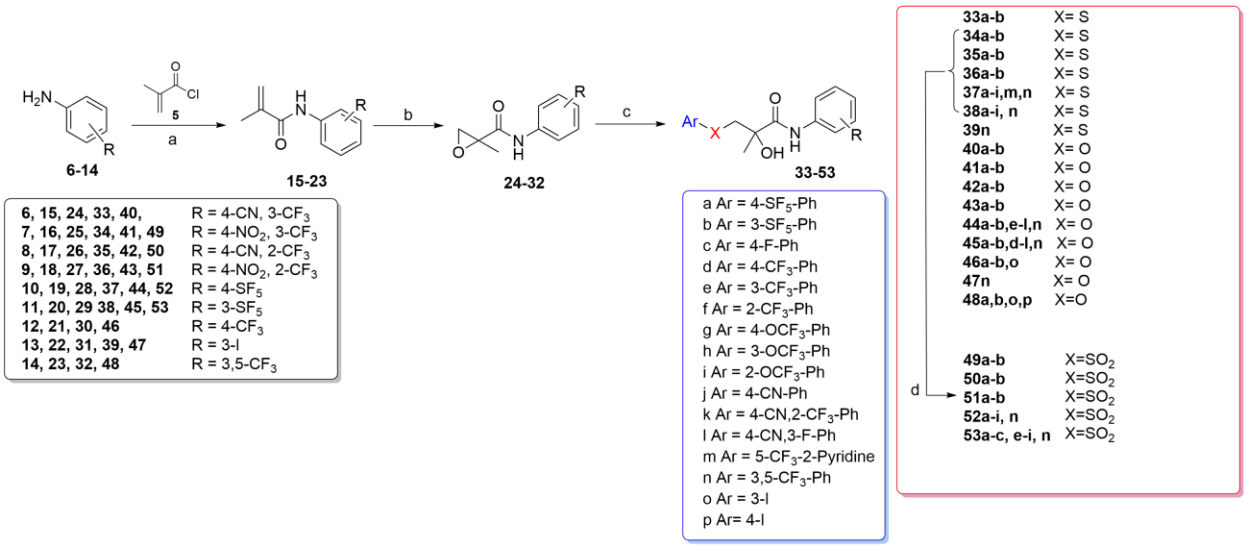
Chemistry

Synthesis of SF_5-substituted bicalutamide and enobosarm derivatives.

The synthesis of bicalutamide and enobosarm-based derivatives have been performed according to our previously reported synthetic strategy (Scheme 1).[57-59] As previously reported by us, racemic and *R*-bicalutamide analogues showed no significant difference in antiproliferative activity and as such, all our enobosarm and bicalutamide-derived structures in this article have been prepared as racemic mixtures. However, R-Bicalutamide was prepared and included in the biological assays as reference.

Reactions of the appropriate functionalised anilines **6-14** (SF_5, CF_3, CN, NO_2 and their combination) with methacryloyl chloride **5** in dimethyl acetamide provided methacrylamides **15**-**23** in good yields (77-95%). These compounds in turn were converted into the epoxides **24**-**32** by oxidation with a hydrogen peroxide/trifluoroacetic anhydride mixture in dichloromethane solution at room temperature for 24 hours. Opening of the epoxide with sodium phenolates/thiophenolates generated by deprotonation with sodium hydride, furnished the desired sulfide/ether derivatives **33**-**53**. Further oxidation of the thioether derivatives with *meta*-chloroperbenzoic acid in dichloromethane solution provided the bicalutamide derivatives **49**-**53** in good yields (57-97%)

Scheme 1. Synthesis of bicalutamide (33-39,49-53) and enobosarm analogues (40-48)



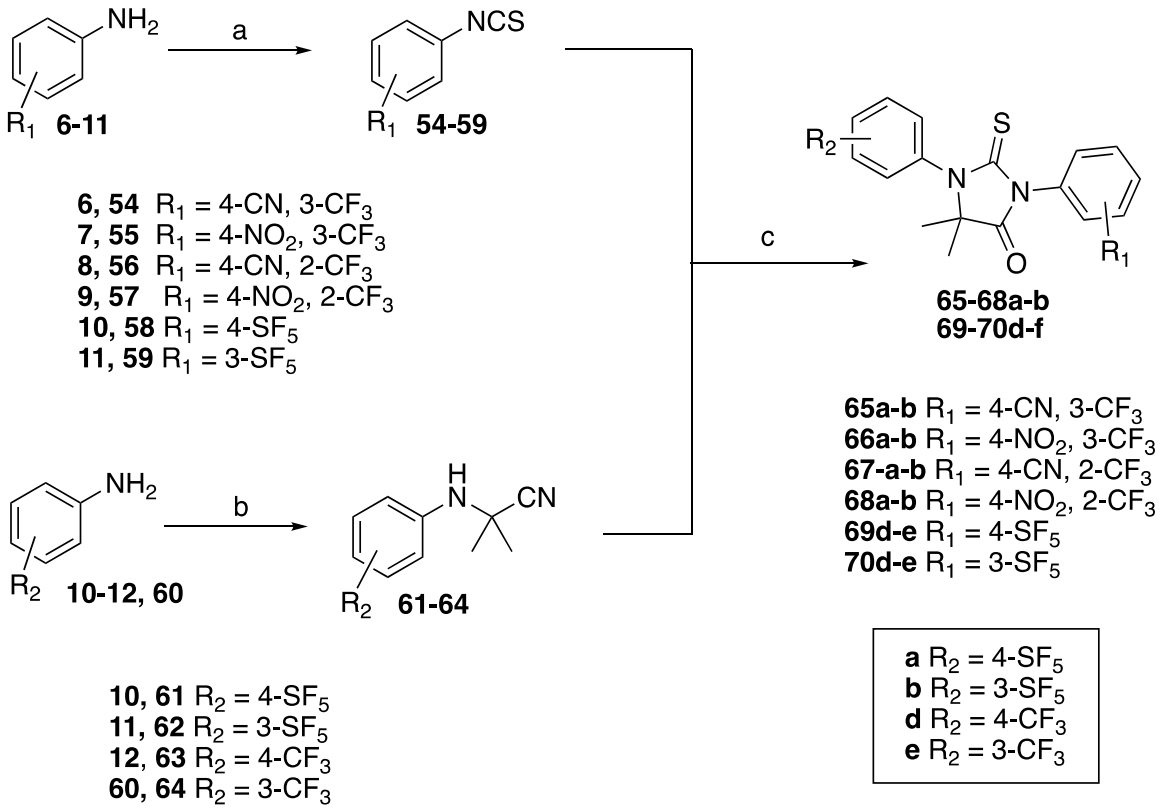
Reagents and conditions: (a) Anilines **6**-**14** (3.4 mmol scale), **5** (8 equiv.), DMA, rt, 3h; (b) **15-23**, H_2O_2 (4 equiv.)/$(CF_3CO)_2O$ (5 equiv.), DCM, rt, 24h; (c) NaH (1.2 equiv.), **24-32** (1.2 equiv.), ArXH **(a-p),** (1.1 equiv.), THF, rt, 24h; (d) *m*-CPBA (1.4 equiv.), DCM, rt, 4-6h.

Synthesis of SF_5 substituted enzalutamide derivatives

Enzalutamide analogues bearing SF_5 group either on B or A ring, were synthesised following two different procedures, both derived by appropriate modification of reported methodologies.[60] Compounds **65**-**68 a,b** and **69**-**70d**-**e** were synthesised according to a three-step literature procedure highlighted in Scheme 2.[61] Briefly, anilines **6-11** were converted into the corresponding isothiocyanates **54-59** by treatment with thiophosgene in dichloromethane solution in presence of sodium bicarbonate. These intermediates were successively reacted with amino-cyanide **61**-**64**, (in turn prepared from anilines **10**-**12** and **60** via Strecker reaction), to obtain final enzalutamide analogues **65**-**68 a**-**b** and **69**-**70d**-**e**. We were also interested in keeping the enzalutamide B ring intact while introducing the pentafluorosulfanyl group on the A ring. The synthesis of these compounds (**75a** and **b**) was accomplished by a literature route reported (Scheme 3).[62] Ullmann type reaction between 4-bromo-2-fluoro-N-methylbenzamide **72** (prepared from the corresponding acid **71**) and 2-aminoisobutyric acid, catalysed by copper chloride, afforded the functionalized aniline **73** in 67% yield. This was converted into the corresponding methyl ester **74** (methyl iodide, potassium carbonate, in a water/dimethylformamide mixture), which was

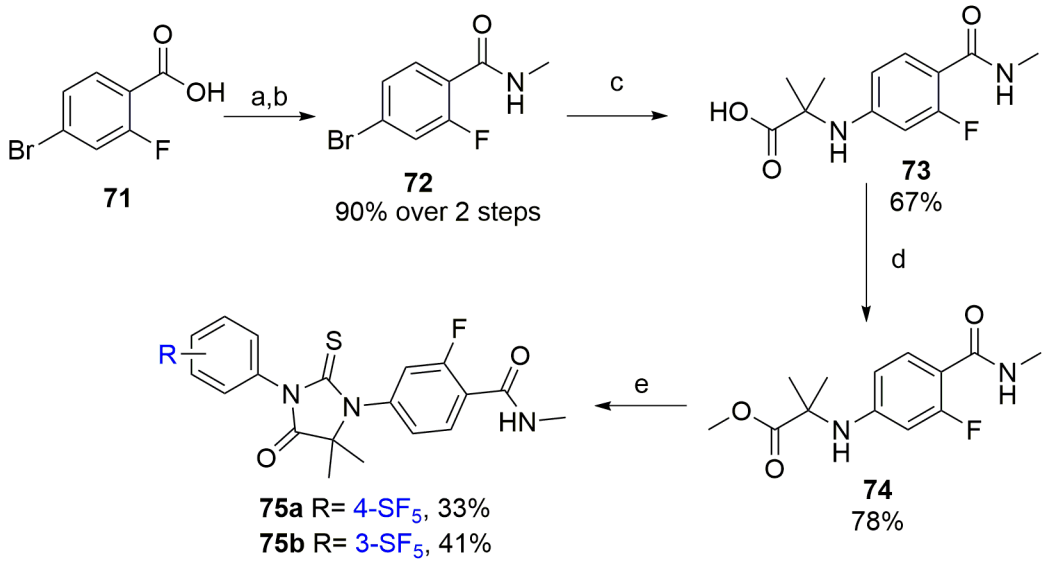
subsequently cyclised into the desired enzalutamide derivatives **75a**-**b** with pentafluorosulfanyl anilines **58** and **59** in hot dimethylsulfoxide/isopropyl acetate mixture for 14 hours.

Scheme 2. Synthesis of enzalutamide derivatives **65-68a-b** and **69-70d-e.**[61]



Reagents and conditions: a) **6**-**11**, $CSCl_2$ (1.5-2 equiv.), $NaHCO_3$ (4.1 equiv.), H_2O, DCM, rt, 24h; b) TMSCN (5.2 equiv.), acetone, 80 °C, 12 h; c) **54**-**59** (1.0 mmol), **61**-**64** (0.84 mmol) DMF, rt, 48 h, then 2N HCl (3 mL), MeOH (10 mL), reflux, 6 h.

Scheme 3. Synthesis of pentafluorosulfanyl enzalutamide derivatives 75a-b.[62]



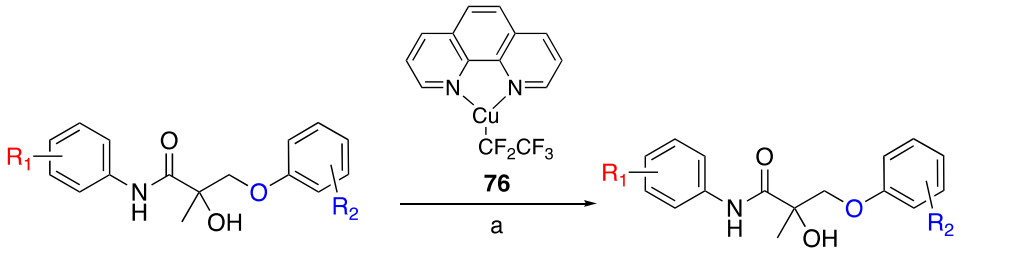
Reagents and conditions: a) $SOCl_2$, iPAc, DMF, 72 °C, 4 h; b) $MeNH_2$, DMF, rt, 15 min; c) 2-aminoisobutyric acid, K_2CO_3, CuCl, 2-acetylcyclohexanone, DMF, H_2O, 105 °C, 14 h; d) MeI, K_2CO_3, H_2O, DMF, 40 °C, 1h; e) **58-59**, DMSO, iPAc, 84 °C, 14 h.

Synthesis of pentafluoroethyl bicalutamides and enobosarms.

As part of our interest in exploring the use of polyfluorinated groups in bicalutamide and enobosarm series, we then moved to investigate the effect of the pentafluoroethyl functional group (C_2F_5), on the anticancer activity of these new derivatives. For the synthesis of these derivatives, we envisaged that an introduction of the desired fluorinated group at the late stage of the synthetic pathway (ideally in the last step) would be the best approach. In the recent years, a great deal of research has been devoted to the introduction of fluoroalkyl group into aromatic rings. Introduction of pentafluoroethyl group onto aromatic compounds have been subjected to a deep investigation and several methods are available in literature, mainly by means of organometallic reagents. These methodologies often require the *in-situ* preparation of the pentafluoethyl-metal reagent[63-65] high temperature (180 ˚C),[63] and careful control of reaction conditions. To avoid these problems, we looked at the work of Hartwig group who have developed a copper-based reagent (pentafluoroethylator®) capable of replacing aromatic iodide or bromide with a CF_3 or a C_2F_5 groups under relatively mild conditions.[66, 67] When we attempted the pentafluorethylation of compounds **46o**, **48o**,**p**, (see Scheme 1), the reaction worked very well and the desired

polyfluorinated derivatives, **77**-**79** were obtained in good yields after chromatographic separation on silica gel (Scheme 4).

Scheme 4. Synthesis of enobosarm's pentafluoroethyl derivatives via pentafluoroethylation of iodides 46o, 48o, 48p.



46o R_1 = 4-CF_3, R_2 = 3-I
48o R_1 = 3,5-CF_3, R_2 = 3-I
48p R_1 = 3,5-CF_3, R_2 = 4-I

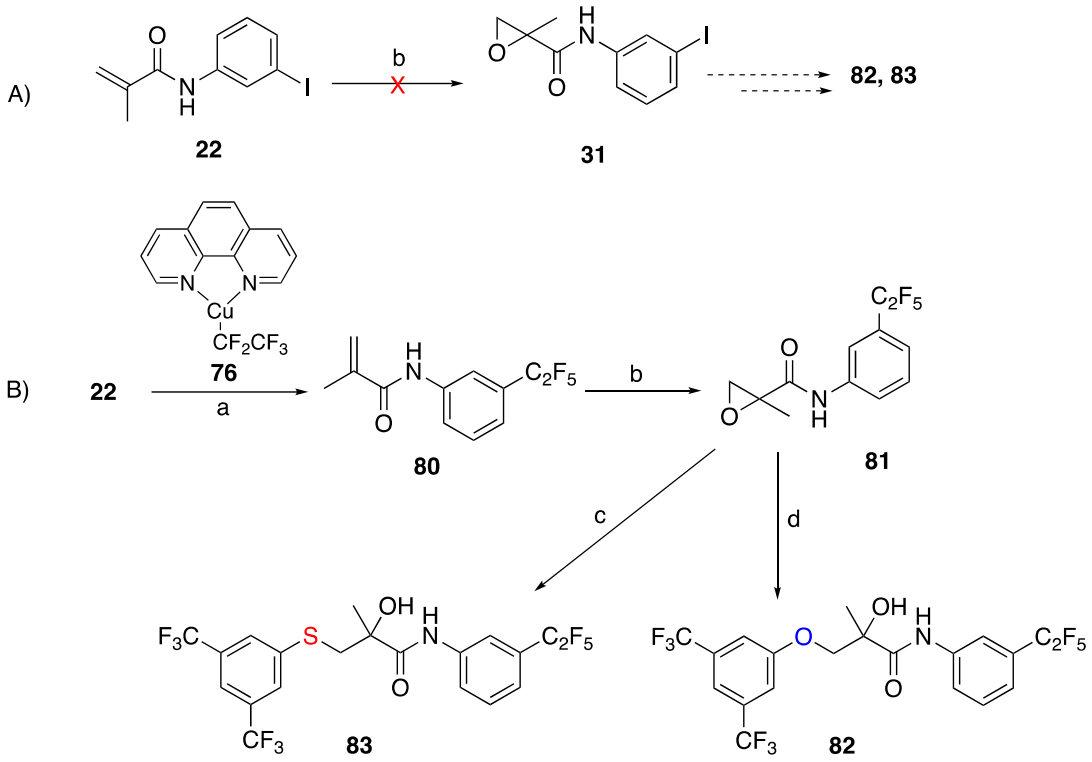
77 R_1= 4-CF_3 R_2 = 3-C_2F_5 60%
78 R_1 = 3,5-CF_3 R_2 = 3-C_2F_5 41%
79 R_1 = 3,5-CF_3 R_2 = 4-C_2F_5 59%

Reagents and conditions: a) Cu(Phen)CF_2CF_3 **76** (2 equiv.), DMF, 50 ˚C, 24 h.

In our previous studies,[53] we demonstrated that Enobosarm analogues with the bis-3,5-CF_3 motif on the ring B were quite active in the antiproliferative assay. We were then interested into investigate if the introduction of the C_2F_5 functionality on ring A of these derivatives could further improve their antiproliferative activity.

However, the synthetic approach proposed in Scheme 1 was unsuccessful for the synthesis these derivatives. In particular, when we attempted the oxidation of 3-iodo alkene **22** with the mixture of trifluoroacetic anhydride and hydrogen peroxide, we were only able to observe trace of the desired product **31** in a complex mixture with other unidentified product likely derived from the formation of hypervalent iodine compounds like bis(trifluoroacetoxy)iodoarene (Scheme 5A).[68] In addition, the presence of the highly electronegative trifluoromethyl group in the ortho position of anilines **8** and **9** made these compounds scarcely reactive, and low yields of the acrylamides **17**, **18** and epoxides **26**, **27** were obtained. We envisaged that this low reactivity would have been even more severe in the case of a C_2F_5 group in this position.

Scheme 5. A) Unsuccessful epoxidation of methacrylamide **22**; B) synthesis of compounds **83** and **82** via pentafluoroethylation of **22**.



Reagents and conditions: a) **76** (2 equiv.), DMF, 50˚C, 48h; b) H_2O_2 (4 equiv.)/$(CF_3CO)_2O$ (5 equiv.), DCM, rt, 24h; (c) NaH (1.2 equiv.) 3,5-bis-trifluoromethyl thiophenol (1.2 equiv.), THF, rt, 24h; d) NaH (1.2 equiv.) 3,5-bis-trifluoromethyl phenol (1.2 equiv.), THF, rt, 24 h.

We therefore decided to attempt the pentafluorethylation directly on compound **22**. When a dry DMF solution of **22** was then treated under argon with pentafluoroethylator **76** (2 equiv.) at 55 ˚C for 48 hours, the desired pentafluoroethyl methacryl amide **80** was obtained in quantitative yield. Epoxidation with trifluoroacetic anhydride and hydrogen peroxide proceeded smoothly affording pentafluoroethyl epoxide **81** which was then coupled with 3,5-bis-trifluoromethyl thiophenol and 3,5-bis-trifluoromethyl phenol to afford compounds **83** and **82** in 27% and 36% yield respectively.

Biological Evaluation

In vitro 2D monolayer antiproliferative assay.

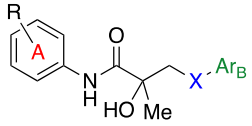
All newly synthesised compounds were evaluated for their antiproliferative effect in an *in vitro* 2D monolayer assay in four human prostate cancer cell lines (LNCaP, 22Rv1, VCaP, and DU145). LNCaP, VCaP and 22Rv1 exhibit androgen sensitivity, whereas DU145 was selected as hormone-insensitive cell line.

Vcap cell line was selected because they show positive response for androgen sensitivity with wild-type AR mRNA/protein, and expresses PSA mRNA/ protein as well.[69] To establish if our compounds are effective also on cells with mutated AR, we selected LNcaP cell line that express endogenous T877A mutation[70, 71] in the AR coding sequence. Finally, 22Rv1 cell line was selected because of endogenously expressed AR splice variants. These have been identified as one of the main players in hormone refractory tumor progression.[72] Two such variants have been identified as specifically a full length isoform with an exon 3 duplication and a C terminal domain truncations with aberrant exon 2b expression.[73]

The antiproliferative activity (absolute IC_{50} in µM) of the most active compounds are reported in table 1 for the bicalutamide/enobosarm series and in table 2 for the enzalutamide series including also the antiproliferative activities of four reference compounds (racemic bicalutamide **1** and its *R*- isomer[74], enzalutamide **2**, enobosarm **40j**), which appeared consistent with previous reported data for these specific cell lines (see supporting information for the complete table of IC_{50s}).

Table 1. Antiproliferative activity of selected polyfluorinated bicalutamide and enobosarm derivatives in four prostate cancer cell lines.

Entry Cpd



X = O Enobosarm derivatives

X = SO_2 Bicalutamide derivatives

	R (A ring)	X	Ar (B ring)	Absolute IC_{50} (µM) [a]

					VCap	LNcap	22Rv1	Du-145
1	**44f**	4-SF_5	O	2-CF_3	3.1	1.2	1.4	7.1
2	**48a**	3,5-CF_3	O	4-SF_5	2.4	1.9	3.2	6.5
3	**44n**	4-SF_5	O	3,5-CF_3	2.6	2.3	7	8.6
4	**79**	3,5-CF_3	O	4-C_2F_5	3.0	2.7	2.5	6.8
5	**45n**	3-SF_5	O	3,5-CF_3	2.8	2.8	6.5	9.3
6	**48b**	3,5-CF_3	O	3-SF_5	2.7	3.0	2.3	5.7
7	**41b**	4-NO_2, 3-CF_3	O	3-SF_5	5.3	3.3	5.4	7.4
8	**83**	3-C_2F_5	S	3,5-CF_3	8.6	3.9	4.2	8.9
9	**77**	4-CF_3	O	3-C_2F_5	8.1	3.9	3.6	7.6
10	**78**	3,5-CF_3	O	3-C_2F_5	9.5	4.4	4.1	8.5
11	**40J**	4-CN, 3-CF_3	O	4-CN	24.5	20.9	24.8	44.6
12	**(1)**	4-CN, 3-CF_3	SO_2	4-F	68.4	45.3	49.6	49.2
13	**R-(1)**	4-CN, 3-CF_3	SO_2	4-F	51.6	45.2	46.3	45.4

[a] All data are mean values from at least triplicate experiments, with standard deviations of ± 10% of the value quoted unless otherwise stated (mean value ± standard deviations).

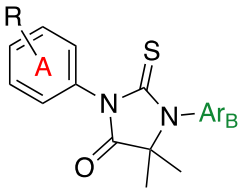
Almost all new derivatives performed better than bicalutamide, showing concentration dependent activity in all four PC in vitro models. It is possible to appreciate that the best linkers between the two portions of the molecule contain either a sulfur or an oxygen atom. When the sulfur was oxidized to the corresponding sulfoxide (or sulfone) a general loss of activity was observed. This is consistent with what has been previously observed with other bicalutamide derivatives.[60, 75] In general, the pentafluorosulfanyl group is slightly more efficient in reducing the IC_{50} when compared with the pentafluoroethyl moiety except when present in the para position of ring B and ring A is functionalised with a 3,5-bistrifluoromethyl moiety (Table 1, entry 4). However, it is important to remark that both polyfluorinated functional groups are more effective than fluorine of CF_3 groups. In terms of ring substitution, from our data, it seems that the SF_5 group is very effective when it is located in the para position of ring A (Table 1, entries 1 and 3) and B (Table 1, entry 2). Compound **44f** (4-SF_5 on ring A and 2-CF_3 on ring B) is more potent than the compound **45f** (full table on SI) with the same substitution pattern on ring B (2-CF_3) but with the SF_5 on ring A moved to the meta position (table 1). However, when the 3,5-bis CF_3 motif is present on either ring A or B the position of the SF_5 (either ortho or meta) on the other ring seem to be not important. Indeed, compounds **48a** and **48b** (table 1 entry 2 vs 6) do shows the same activities except in LNCaP cell where **48a** seem to perform better. In the pentafluoroethyl series it is clear from the data of table 1 that p-C_2F_5 in the B ring is very effective for the antiproliferative activity. When the ring A is substituted with the 3,5-bis-trifluoromethyl moiety, moving the pentafluoroethyl moiety from the 4-position to the 3-position resulted in the reduction of activity (Table 1 entries 4 vs 10).

Considering the antiproliferative results across the four different cell lines, the new compounds showed significant activity also on androgen-insensitive DU-145 cells with IC_{50} values for most compounds higher in this cell line than in the other three, suggesting the presence of a potential off-target effect, besides the canonical anti-androgen mechanism. Interestingly, some studies have demonstrated that even DU-145 and PC3 cell lines are expressing significant levels of AR protein, although at a lower level than LnCaP cell line.[76] In addition, DU-145 cell line usually express NF-kB, a transcription factor involved in prostate cancer progression.[77] It seem plausible to speculate that our molecules are either blocking the AR expressed by the DU-145 cells or are somewhat able to target other pathways such as the expression of NF-kB.

Within the enzalutamide series only three derivatives (**67a, 70e** and **67b)** showed a better activity than enzalutamide itself (Table 2) in LnCaP cell line. Unfortunately, in these series, the solubility

of these polyfluorinated compounds posed a serious limitation in their anti-proliferative evaluation (See SI for complete table of the antiproliferative data).

Table 2. Antiproliferative activity of selected polyfluorinated enzalutamide derivatives in four prostate cancer cell lines.

Entry	Cpd						
		R (A ring)	**Ar (B ring)**	**Absolute IC_{50} (µM)[a]**			
				VCap	**LNcap**	**22Rv1**	**Du-145**
1	**67a**	4-CN, 3-CF_3	4-SF_5	3	5.4	12.7	3
2	**70e**	3-SF_5	3-CF_3	3.4	ND	ND	3.4
3	**67b**	4-CN, 2-CF_3	3-SF_5	4.1	ND	ND	4.1
7	**2**	4-CN, 3-CF_3	3-F,	11.5	31.8	32.3	11.5
	Enzalutamide		4-CONHMe				

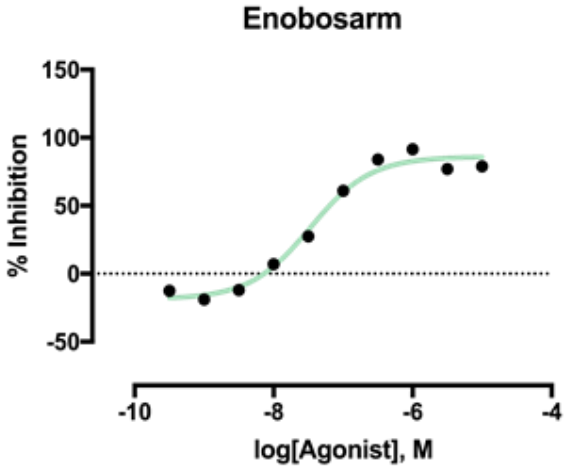
[a] All data are mean values from at least triplicate experiments, with standard deviations of ± 10% of the value quoted unless otherwise stated (mean value ± standard deviations).

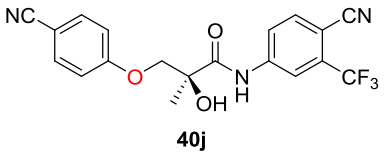
During the preparation of this manuscript, a similar study appeared in the literature where pentafluorosulfanyl substituted enzalutamide analogues, were evaluated for their *in vitro* efficacy in LnCaP cell lines as well as for their AR antagonism.[78] Similar to our findings, the antiproliferative activity of these derivatives, although structurally different from ours, was found similar to enzalutamide. In addition, antagonism behaviour was proved for these compounds.

Agonist-antagonism assay

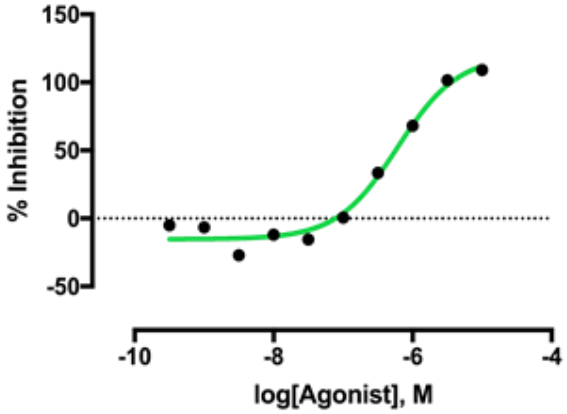
Once established the efficacy of these new derivatives in the antiproliferative assay, we progressed to evaluate their AR antagonist activity.

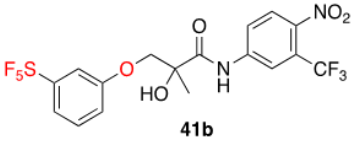
Among all the derivatives prepared, compound **44f** with a SF_5 group in the A ring and our best analogue in terms of antiproliferative activity, along with compound **41b,** bearing the pentafluorosulfanyl substituent on the B ring were investigated in an agonist/antagonist assay (Figure 4). We used the GeneBLAzer® beta lactamase reporter technology adapted to the nuclear receptor, to asses if these compounds are AR antagonists. Indeed, we observed that both compounds behave as full AR antagonists. It is worth noting that introduction of the SF_5 motif leads to the switch of the enobosarm scaffold from partial agonist to full antagonist of the AR receptor, as also previously observed by us with other fluorinated groups.[53, 60] Figure 4 below shows the dose response curve for enobosarm **40j** (A) **41b** (B) and **44f** (C). Table 3 collects the IC_{50} values, the antagonistic and agonistic mean effect, expressed as percentage, for the three compounds in the same assay. This result is of great significance for the future development of AR antagonist and ultimately for the treatment of PC condition.


Enobosarm
% Inhibition
150
100
50
0
-50
-10
-8
-6
-4
log[Agonist], M

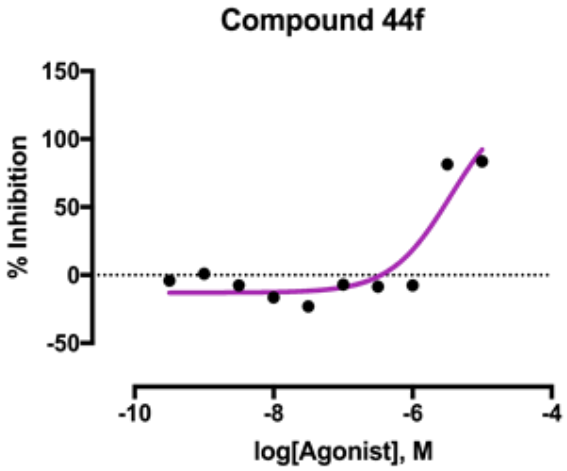

NC
O
CN
O
N
H
CF3
OH

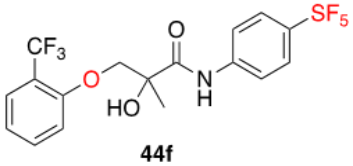
40j


Compound 41b
% Inhibition
150
100
50
0
-50
-10
-8
-6
-4
log[Agonist], M


NO2
O
F5S
O
N
H
CF3
HO

41b


Compound 44f
% Inhibition
150
100
50
0
-50
-10
-8
-6
-4
log[Agonist], M


SF5
CF3
O
O
N
H
HO

44f

Figure 4. Dose Response curve (at 10 different concentrations, max 10 μM) for the antagonist assay for Enobosarm **40j** (A) and compounds **41b** (B), **44f** (C). The mean value from the two experiments has been plotted in a graph log (antagonist) ([M]) against % of inhibition.

Table 3. AR antagonistic and agonistic activity of enobosarm (**40j**)**,** compounds **41b, 44f** and, as comparison, Bicalutamide (**1**).

Cpds	**Antagonistic (mean % inhibition)**[1,2,3]	**IC_{50} (μM)**[1,3]	**Agonistic (mean % activation)**[1,4]
40j	59	0.036	26
41b	90	0.540	2
44f	93	1.810	-3
1	83	0.490	5

[1]Data presented are the means from duplicate experiments; [2]Compounds were considered full antagonists if at 10μM the reduction of R1881 (standard AR antagonist) effect was greater than 80%; [3]Data from compounds tested at 10 different concentrations; [4]Data from compounds tested at single (10 μM) concentration in absence of R1881.

In vitro Absorption, Distribution, Metabolism, and Excretion (ADME) screening.

Compounds **44f**, **41b** and **48a** were evaluated for their metabolic stability, binding to protein and cardiotoxicity (Table 4). First the tested compounds were incubated for 45 min with pooled liver microsomes, and the intrinsic clearance (CL_{int}) and half-life ($t_{1/2}$) values were calculated based on 5 time points for a maximum of 45 minutes (Table 4). All compounds examined, except **44f** (which shows a medium CL_{int} value and short half-life), have low intrinsic clearance in liver microsome and long half-lives (~7.5 hours). In particular, compounds **48a**, **41b**, showed CL_{int} values below 6

µL/min/mg microsomal protein, suggesting that these compounds will undergo much slower rates of hepatic metabolism *in vivo*. In addition, compounds **48a** and **41b** showed a metabolic stability greater than bicalutamide confirming the reported bio-stability of the pentafluorosulfanyl motif.[79] All the selected candidates showed plasma protein binding greater than 91%. Our most active compound **44f**, showed a 98.6% protein binding very similar to the bicalutamide value (96%).[80] The least bound to plasma protein was compound **48a** (91%). Moreover, all tested compounds lacked significant hERG inhibition in the patch-clamp assay (at 6 different concentrations). In particular, the most active compounds in antiproliferative assay, **44f** and **48a** showed a mean hERG inhibition of 26.3% and 13.4% respectively at 25 µM. The IC_{50} calculated over 6-different concentrations were all above 25 µM showing a satisfactory degree of safety for these derivatives.

Table 4. Metabolic stability in pooled liver microsomes, plasma protein binding and cardiotoxicity results of selected compounds.

Comp	CL_{int}[a,b]	$T_{1/2}$ (min.)	PPB (% bound)[b]	hERG (% mean inhibition)[c]
44f	22.0	63.1	98.6	26.3
48a	3.08	450	90.9	13.4
41b	3.21	431	98.5	38.1
(**R)-1**	6.48	214	98[d]	ND[e]

[a] µL/min/mg protein, from quadruplicate assay; [b] Data expressed as mean values from duplicate experiments; [c] data at 25 µM: Weak or no inhibition from quadruplicate experiments if IC_{50} >10µM; [d] From literature data;[80] [e] Not Determined.

In vivo efficacy evaluation in 22Rv1 prostate cancer model.

With the ADME data in hand we decided to evaluate one of our best performing pentafluorosulfanyl derivatives in an *in vivo* efficacy xenograft model in mice to assay their ability to impair the growth of prostate tumour in comparison to the standard-of-care docetaxel. Among

the most active bicalutamide/enobosarm series in the antiproliferative activity, compound **48a**, although slightly less potent than compound **44f** was selected for in *vivo evaluation* because its longer $t_{1/2}$ value, and lower plasma protein binding and because it was one of the better performing compound in our previously published results on bis-CF_3 analogues.[53]

At first, the tolerability of **48a** were tested in nude, non-tumour bearing mice with the maximum tolerated dose (MTD) established at 100 mg/Kg and then used later for the xenograft experiment. Standard-of-care docetaxel was instead given at a dose level of 15mg/Kg. (Table 5).

Table 5. Summary of the *in vivo* efficacy experiments Tumor Model PRXF 22Rv1

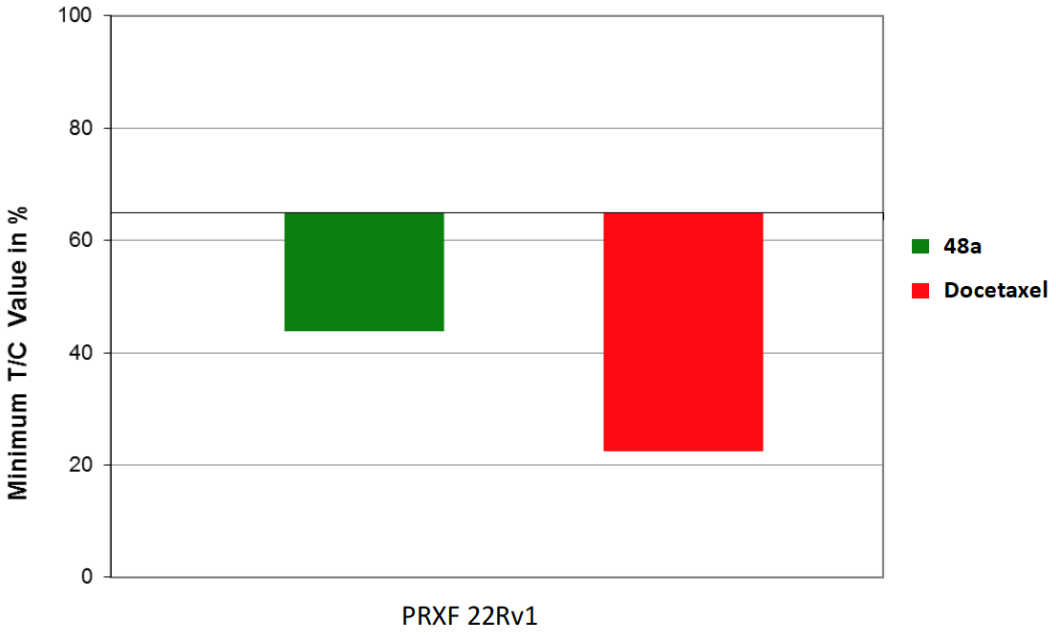
Therapy[a]	Dose Level [mg/kg/day]	Schedule [dosing Days]	Route	Minimum T/C [%] (Day)[b]	Efficacy Rating	Td [Days][c]	Tq [Days][d]
Vehicle[e]	10 ml/kg/day	0-18	i.p.	n/a	n/a	2.7	6.2
48a	100	0-16	i.p.	43.9 (14)	+	6.7	14.3
Docetaxel[e]	15	0,7,14	i.v.	22.4 (14)	++	6.3	43.2

n/a, not applicable; n.r., not reached (i.e. group median RTVs always < 200% / 400%); Efficacy rating: ++++, T/C < 5%, +++, T/C: 5 - < 10%; ++, T/C: 10 - <25%; +, T/C: 25 - <50%; +/-, T/C : 50-65%; -, T/C ≥ 65%; [a] Vehicle for **48a** and the control group: 10% EtOH, 10% Cremophor EL, 5% dextrose; vehicle for docetaxel, 0.9% Saline; [b] Minimum T/C values are calculated based on median values; [c] Tumor volume doubling time; [d] Tumor volume quadrupling time; [e] Docetaxel was used as positive control, while DMSO (vehicle) was used as negative control.

Then, *in vivo* efficacy data were obtained selecting 22Rv1 cells to perform this study. Mice were injected in the hind flank with tissue culture 22Rv1 cells. Once enough tumors have formed, the tumor-bearing mice were randomised into groups and therapy with **48a** and docetaxel were performed for 4 weeks. Treatment with the investigational compound was given as 16-18 daily intraperitoneal (i.p.) doses, whereas docetaxel was given as three weekly intravenous (i.v.)

treatments. The treatment period was followed by an observation period until day 40 for **48a** group and until day 49 in the docetaxel group. The first day of dosing was either the day of randomisation (day 0) or the following day (day 1). Anti-tumor efficacy was evaluated as inhibition of tumor growth represented by minimum T/C (treated to control) value based on group median relative tumor volumes (RTVs) in test and control groups (see SI for the pertinent plot). The body weight was also recorded to assess the animal's tolerance of the compound. The group receiving treatment with **48a** was compared to the docetaxel and to the control groups. The highest anti-tumor efficacy was observed in the docetaxel-treated group with a minimum T/C value of 22.4%. Compound **48a** displayed moderate anti-tumor efficacy with a minimum T/C value of 43.9%. (Figure 5) Statistical significance was observed for both docetaxel and compound **48a** in comparison to the vehicle control group.

Figure 5. Efficacy of compound **48a** and docetaxel in R22v1 xerograph mice model.



Minimum T/C values for the two groups are shown. As a turning point, a minimum T/C value of 65% (upper limit for borderline anti-tumor efficacy) was chosen.

As it can be appreciated by the figure 6A that in the first 10 days of administration, compound **48a** was able to better control the absolute volume tumor growth with respect to Docetaxel which

become more effective as the time progress to 20 days.Treatment with **48a** was well-tolerated with adjusted survival rates of 100% and no group median body weight losses (BWLs, Figure 6B). A severe BWL of 19.8% was recorded in the docetaxel-treated group, although the adjusted survival rate was 100%.

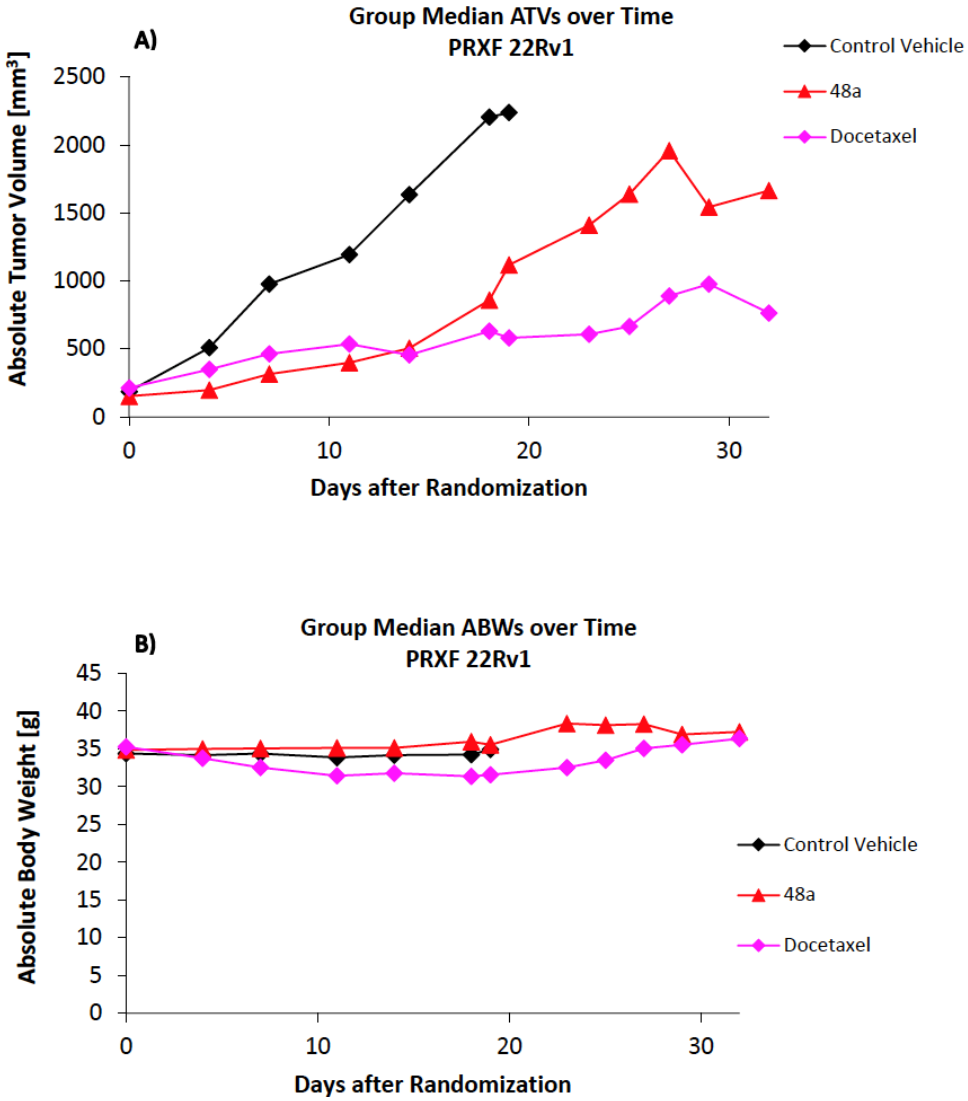


Figure 6. Plot of medians absolute (A) tumor volume and (B) body weight for **48a** and docetaxel as function of time.

Conclusions

In summary, we have demonstrated that functionalisation of bicalutamide and enobosarm scaffold with pentafluorosulfanyl and pentafluoroethyl functionality, lead to the development of potent antiproliferative agents against prostate cancer in *in vitro* models. The IC_{50} for the majority of the newly prepared molecules were about 48-times better than the parent drugs. Among the compounds with improved anticancer activity, enobosarm analogues performed better with respect to their bicalutamide analogues, pointing out that a less bulky linker (compared to SO_2) between ring A and B lead to a better activity.

Overall SF_5 structures displayed the best *in vitro* anticancer activity, together with AR full antagonism (a key requirement for a drug against prostate cancer) and ADME properties. One of the most active compounds (**48a**) showed also some efficacy in *in vivo* xenograft experiment, although its activity was lower than the standard of care docetaxel. Pleasingly, (**48a**) was better tolerated with respect to docetaxel as evidenced by the lack of body weight lost in the treated mice. Pentafluoroethyl derivatives also showed an excellent improvement of the antiproliferative activity of the parent compound.

In contrast, SF_5-functionalised Enzalutamides were hampered by low solubility that precluded their full evaluation in animal model, although one derivative (**67a**) showed an improved antiproliferative activity (*in vitro*) with respect to enzalutamide. Altogether, our results are indicative of the fact that both SF_5 and C_2F_5 are indeed two functionalities that can be used in place of the trifluoromethyl group and exploited for the design of drug-like molecules.

Having established in this work that the *in vitro* anticancer activity can be extensively improved, for a successful progression of these derivatives to clinical studies, further evaluation of their biological properties (PC protein expression, inhibition of AR-mediated gene transcription, quantitative AR binding assay) needs to be addressed. These experiments are currently underway in our laboratories and will be reported in due course.

Experimental

General Chemistry methods

All solvents and reagents were used as obtained from commercial sources unless otherwise indicated. All reactions were performed under a nitrogen atmosphere. The 1H and 13C NMR spectra were recorded on a Varian spectrometer operating at 500 MHz for 1H and 125 MHz for 13C. Deuterated chloroform was used as the solvent for NMR experiments, unless otherwise stated. 1H chemical shifts values (δ) are referenced to the residual nondeuterated components of the NMR solvents (δ = 7.26 ppm for $CHCl_3$, etc.). The 13C chemical shifts (δ) are referenced to $CDCl_3$ (central peak, δ = 77.0 ppm). Fluorine chemical shifts are referenced to $CFCl_3$. Mass spectra were measured in positive mode electrospray ionization (ESI). TLC was performed on silica gel 60 F254 plastic sheets. Column chromatography was performed using silica gel (35–75 mesh) or on an Isolera Biotage system. Purity of prepared compounds was determined by HPLC-UV analysis (Thermo HPLC connected with UV detector). The purity of all final compounds was determined to be >95% by RP-HPLC using the eluents water (eluent A), acetonitrile (eluent B), and the following conditions: Varian Pursuit XS, 4.6 mm × 150 mm, 5.0 μm, 1.0 mL/min, gradient 30 min 10% → 100% eluent B in eluent A (method 1). HRMS were acquire on a Waters XEVO-27-Jun-2019 G2XSQTOF instrument under electrospray condition in both positive and negative modes.

Synthetic Procedures

General method for the preparation of intermediates **15**-**23, 80**

Methacryloyl chloride **5** (2.63 mL, 27.16 mmol) was added over the course of 10 minutes to a stirring solution of the different aniline 6-14 (3.4 mmol) in N,N-dimethylacetamide (14 mL) at r.t. for 3 h or o.n. After the reaction was complete, the mixture was diluted with ethyl acetate (100 mL), extracted with sat. aq. $NaHCO_3$ solution (3x25 mL) and with cold brine (4x50 mL). The organic layer was dried over Na_2SO_4 and the solvent vas removed at reduced pressure. The crude residue was purified by flash column chromatography.

N-(4-(Pentafluorosulfanyl)phenyl)methacrylamide **19**

Purified by flash column chromatography eluting with *n*-hexane-EtOAc 100:0 v/v increasing to *n*-hexane-EtOAc 80:20 v/v. Obtained in 99% yield as a white solid. ^{1}H NMR ($CDCl_3$), δ: 7.74 (d, J = 9.2 Hz, 2H), 7.70-7.68 (m, 3H), 5.84 (s, 1H), 5.56-5.55 (m, 1H), 2.09 (m, 3H). ^{19}F NMR ($CDCl_3$), δ: 85.3 (quintet, J = 151.1 Hz, 1F), 63.5 (d, J = 151.1 Hz, 4F). ^{13}C NMR ($CDCl_3$), δ: 166.7, 140.2 (m), 127.0 (m), 120.8, 119.1, 103.9, 80.7, 30.1, 18.6.

N-(3-iodophenyl)methacrylamide **22**

Purified by flash column chromatography eluting with *n*-hexane-EtOAc 100:0 v/v increasing to *n*-hexane-EtOAc 90:10 v/v. Obtained in 60% yield as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.00 (s, 1H, NH), 7.57 (ddd, J = 0.7, 2.0, 8.2 Hz, 1H), 7.48 (ddd, J = 1.0, 1.5, 7.9 Hz, 1H), 7.08 (t, J = 8.0 Hz, 1H), 5.81 (s, 1H), 5.51 (d, J = 1.4 Hz, 1H), 2.08 (d, J = 0.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.50, 140.65, 138.90, 133.45, 130.47, 128.72, 120.21, 119.21, 103.99, 94.08, 80.81, 30.11, 24.18, 18.46.

N-(3,5-bis(Trifluoromethyl)phenyl)methacrylamide **23**

Purified by flash column chromatography eluting with n-hexane-EtOAc 100:0 v/v increasing to n-hexane-EtOAc 90:10 v/v. Obtained in 99% yield as a white solid. ^{1}H NMR ($CDCl_3$), δ: 8.11 (s, 2H), 7.76 (bs, 1H), 7 1H), 5.87 (d, *J* = 1.1 Hz), 5.59 (m, 1H), 2.1 (s, 3H). ^{19}F NMR ($CDCl_3$), δ: -63.0 (s, 3F). ^{13}C NMR ($CDCl_3$), δ: 166.6, 140.2 (q, *J* = 33.6 Hz), 139.2, 137.5 (q, *J* = 272.8 Hz), 121.0, 117.6 (m), 18.6.

Preparation of N-(3-pentafluoroethyl)phenyl)methacrylamide **80**

A solution of iodide **22** (0.8 g, 2.8 mmol), and (1, 1, 2, 2, 2- Pentafluoroethyl) (1, 10-phenanthroline- κN1, κN10)-copper (Pentafluoroethylator®, 5.6 mmol, 2 equiv) in dry DMF where heated at 50 ˚C for 48 hours. After this period the mixture was cooled at room temperature and diluted with diethyl ether and the orange solution filtered through a pad of celite. The pad was washed with diethyl ether and the combined organic were washed with 2M HCl (2x 10 mL) sat $NaHCO_3$ (2x 10 mL) dried and evaporated. The residue was pure enough (90%) to be used in the next step.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (t, *J* = 12.0 Hz, 2H), 7.65 (s, 1H), 7.50 (t, *J* = 8.0 Hz, 1H), 7.38 (d, *J* = 7.8 Hz, 1H), 5.85 (s, 1H), 5.55 (d, *J* = 1.4 Hz, 1H), 2.10 (s, 3H). ^{19}F NMR ($CDCl_3$), δ: -84.63 (s), -117.01 (br s); MS [ESI, m/z]: 302.05 [M+Na].

General method for the preparation of intermediates 24-32

To a stirred solution of the different intermediate **15**-**23** (3 mmol) in DCM (7 mL) was added 30% hydrogen peroxide (3.6 mL, 32.03 mmol). The reaction mixture was put in a

water bath at r.t. and trifluoroacetic anhydride (3.7 mL, 26.7 mmol) was added slowly to the mixture, which was then stirred for 24 h. The reaction mixture was transferred to a separating funnel using DCM (30 mL). The organic layer was washed with distilled water (20 mL), sat. aq. $Na2S_2O_3$ (4x20 mL), sat. aq. $NaHCO_3$ (3x20 mL) and brine (20 mL), dried over Na_2SO_4 and concentrated at reduced pressure. These intermediates were usually of good purity (90%) to be used in the next step without further purification. When necessary, column chromatography (EtOAc/Hexane, silica gel) or preparative TLC were used to purify the compounds.

N-(4-(pentafluorosulfanyl)phenyl)-2-Methyloxirane-2-carboxamide ***28***

Obtained in 73% yield as a white wax. ^{1}H NMR ($CDCl_3$), δ: 8.32 (bs, 1H), 8.03-8.02 (m, 1H), 7.72-7.70 (m, 1H), 7.52-7.49 (m, 1H), 7.44-7.39 (m, 1H), 3.01 (d, *J* = 4.2 Hz, 1H), 2.97 (d, 4,8 Hz, 1H), 1.67 (s, 3H). ^{19}F NMR ($CDCl_3$), δ: 85.1 (quintet, *J* = 151.1 Hz, 1F), 63.0 (d, *J* = 151.1 Hz, 4F). ^{13}C NMR ($CDCl_3$), δ: 169.0, 154.0 (p, *J* = 18.4 Hz), 137.6, 129.2, 122.6, 121.9 (p, *J* = 4.8 Hz), 117.3, 67.6, 56.6, 16.6; MS [ESI, m/z]: 326.2 [M+Na].

N-(3-(pentafluorosulfanylphenyl)-2-Methyloxirane-2-carboxamide ***29***

Obtained in 72% yield as a white solid. ^{1}H NMR ($CDCl_3$), δ: 8.05-7.99 (m, 1H), 7.82-7.77 (m, 1H), 7.72 (bs, 1H), 7.55-7.50 (m, 1H), 7.48-7.40 (m, 1H), 5.85 (s, 1H), 5.55 (m, 1H), 2.09 (m, 3H). ^{19}F NMR ($CDCl_3$), δ: 83.96 (quintet, *J* = 151.2 Hz, 1F), 62.62 (d, *J* = 151.2 Hz, 4F). ^{13}C NMR ($CDCl_3$), δ: 166.7, 140.4 (p, *J* = 18.1 Hz), 138.1, 129.2, 122.9, 121.7 (p, *J* = 4.4 Hz), 120.6, 117.6 (p, *J* = 4.5Hz), 60.4, 18.6.

N-(3,5-bis(Trifluoromethylphenyl)-2-methyloxirane-2-carboxamide **32**

Obtained in 82% yield as a white solid. ^{1}H NMR ($CDCl_3$), δ: 8.35 (bs, 1H), 8.07 (s, 2H), 7.64 (s, 1H), 3.02 (m, 2H), 1.71 (s, 3H). ^{19}F NMR ($CDCl_3$), δ: -63.1 (s, 3F). ^{13}C NMR ($CDCl_3$), δ: 169.0, 138.4, 132.6 (m), 119.3, 117.8 (m), 56.6, 16.7.

General method for the preparation of compounds 33-48

To a mixture of NaH (60% in mineral oil, 0.050 g, 1.23 mmol) in anhydrous THF (2 mL) at 0 °C under Ar atmosphere was added a solution of the differently substituted phenol or thiophenol (1.11 mmol) in 1 mL of anhydrous THF. This mixture was stirred at r.t. for 20 minutes. A solution of the different intermediate **24**-**32** (0.74 mmol) in anhydrous THF (3 mL) was added slowly. The reaction mixture was stirred at r.t. overnight. The mixture was then diluted with ethyl acetate (30 mL), washed with brine (15 mL), water (30 mL), and dried over Na_2SO_4 and concentrated under vacuum. The crude residue was purified by flash column chromatography or via Biotage Isolera 1.

N-(4-(pentafluorosulfanyl)phenyl)-3-(2-(trifluoromethyl)phenoxy)-2-Hydroxy-2-methyl propanamide **44f**

Purified by flash column chromatography eluting with n-hexane/EtOAc 100:0 v/v increasing to n-hexane/EtOAc 70:30 v/v. Obtained in 59% yield as a white solid. ^{1}H NMR ($CDCl_3$), δ: 8.84 (bs, 1H), 7.75 (d, J = 9.2 Hz, 2H), 7.69 (d, J = 9.2 Hz, 2H), 7.61-7.59 (m, 1H), 7.56-7.53 (m, 1H), 7.12-7.09 (m, 1H), 7.03 (d, J = 8.3 Hz, 1H), 4.48 (d, J = 8.7 Hz, 1H), 4.12 (d, J = 8.7 Hz, 1H), 3.55 (s, 1H), 1.64 (s, 3H). ^{19}F NMR ($CDCl_3$), δ: 84.8 (quintet, J = 150.1 Hz, 1F), 62.9 (d, J = 150.1 Hz, 4F), -61.8 (s, 3F). ^{13}C NMR ($CDCl_3$), δ: 172.2, 155.3, 149.5 (m), 139.8, 133.7, 127.2 (m), 127.0 (m),

124.8, 122.6, 121.3, 120.5, 119.2 (m), 118.5, 112.9, 75.1, 72.9, 22.8. MS [ESI, m/z]: 488.0 [M+Na]. HPLC (method 1): retention time = 24.60 min.

N-(3,5-(bis-trifluoromethyl)phenyl)-3-(4-pentafluorosulfanylphenoxy)-2-hydroxy-2-methyl-propanamide **48a**

Purified by flash column chromatography eluting with n-hexane/EtOAc 100:0 v/v increasing to n-hexane/EtOAc 50:50 v/v. Obtained in 54% yield as a thick transparent oil. ^{1}H NMR (500 MHz, $CDCl_3$) *δ*: 9.09 (s, 1H), 8.15 (s, 2H), 7.22-7.66 (m, 3H), 6.96 (d, *J* = 9.1 Hz, 2H), 4.53 (d, *J* = 9.1 Hz, 1H), 4.08 (d, *J* = 9.1 Hz, 1H), 3.40 (s, 1H), 1.65 (s, 3H). ^{19}F NMR ($CDCl_3$), δ: 85.36 (quintet, *J* = 151.1 Hz, 1F), 64.08 (d, *J* = 151.1 Hz, 4F), -62.9 (s, 1F). ^{13}C NMR (126 MHz, $CDCl_3$) *δ:* 172.1, 159.5, 147.61 (t, *J* = 18.0 Hz), 138.60, 132.53 (q, *J* = 33.6 Hz), 127.83 (d, *J* = 8.9 Hz), 123.02 (q, *J* = 272.8 Hz), 119.37 (d, *J* = 3.2 Hz), 117.96 (d, *J* = 7.6 Hz), 75.69, 72.71, 23.9; MS [ESI, m/z]: 534.05 [M+H], 556.05 [M+Na]. HPLC (method 1): retention time = 26.39 min.

General method for the preparation of isothiocyanates **54**-**59**

A solution of $NaHCO_3$ (0.82 g) in distilled water (4 mL) was stirred for 10 minutes and to it was added DCM (4 mL) followed by the different aniline 7-12 (2.4 mmol). The reaction mixture was cooled to 0 °C, thiophosgene (0.3 mL, 3.7 mmol) was added dropwise over 10 minutes and the reaction was then stirred at r.t. overnight. The mixture was then diluted with DCM (20 mL), washed with brine (20 mL), the organic layer was dried over Na_2SO_4 and concentrated to dryness.

4-Isothiocyanato-3-(trifluoromethyl)benzonitrile **56**

Aniline **9** (4.9 mmol) was dissolved in 9 mL toluene. Thiophosgene (10.67 mmol) was added dropwise and the mixture was stirred at 75 °C for five days. Obtained in 99% yield as a brown wax. ^{1}H NMR ($CDCl_3$), δ: 7.97 (d, *J* = 1.6 Hz, 1H), 8.86 (dd, J_1= 8.4 Hz, J_2=

1.6 Hz, 1H), 7.53 (d, *J* = 8.4 Hz, 1H). ^{19}F NMR ($CDCl_3$), δ: -63.12 (s, 3F). ^{13}C NMR ($CDCl_3$), δ: 136.4, 130.9 (q, *J* = 5.5 Hz), 129.3, 122.7 (m), 120.5, 119.1 (m), 117.1, 116.6, 110.5.

General method for the preparation of nitriles **61**-**64**

The different substituted anilines **10**-**12** and 60 (2.2 mmol) was added to a TMSCN solution (11.4 mmol) in acetone (10 mL). The mixture was heated at 80 °C overnight. The reaction was then concentrated in vacuo and the residue was partitioned between water (15 mL) and ethyl acetate (15 mL). The water phase was re-extracted with ethyl acetate (2x15 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 and concentrated to dryness.

2-Methyl-2-((4-(pentafluorosulfanyl)phenyl)amino)propanenitrile **61**

Obtained in 69% yield as white crystals. ^{1}H NMR ($CDCl_3$), δ: 7.53 (d, *J* = 9.0 Hz, 2H), 6.63 (d, *J* = 9.0 Hz, 2H), 4.03 (bs, 1H), 1.62 (s, 6H). ^{19}F NMR ($CDCl_3$), δ: 86.4 (quintet, *J* = 150.1 Hz, 1F), 64.6 (d, *J* = 150.1 Hz, 4F). ^{13}C NMR ($CDCl_3$), δ: 146.0, 145.6 (p, *J* = 17.5 Hz), 127.4 (p, *J* = 4.8 Hz), 120.9, 114.1, 48.0, 28.0.

General method for the preparation of thiohydantoins **65**-**70**

A mixture of the different isothiocyanate **54**-**59** (1.01 mmol) and the different nitrile **61**-**64** (00.84 mmol) in dry DMF (0.5 mL) was stirred at r.t. for 48 h. To this mixture were added MeOH (10 mL) and 2N HCl (3 mL). The second mixture was refluxed for 6 h. After being cooled to r.t., the reaction mixture was poured into cold water (20 mL) and extracted

with ethyl acetate (20 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude residue was purified by flash column chromatography.

4-(4,4-dimethyl-5-oxo-2-thioxo-3-(4-pentafluorosulfanyl)phenyl)imidazolidin-1-yl)-2-(trifluoromethyl)benzonitrile **67a**

Purified by flash column chromatography eluting with *n*-hexane/EtOAc 100:0 v/v increasing to *n*-hexane/EtOAc 70:30 v/v. Obtained in 25% yield as a white solid. ^{1}H NMR ($CDCl_3$), δ: 8.01 (d, J = 8.3 Hz, 1H), 7.99-7.96 (m, 3H), 7.86 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H), 7.47 (d, J = 8.7 Hz, 2H), 1.64 (s, 6H). ^{19}F-NMR ($CDCl_3$), δ: 82.68 (quintet, J = 150.9 Hz, 1F), 62.94 (d, J = 150.9 Hz, 4F), -61.99 (s, 3F). ^{13}C NMR ($CDCl_3$), δ: 179.8, 174.4, 154.1 (m), 138.1, 136.8, 135.2, 133.6 (q, J = 33.6 Hz), 132.1, 130.2, 127.8 (m), 127.0 (q, J = 4.9 Hz), 120.7 (q, J = 272.0), 110.5 (m), 66.5, 23.8. MS [ESI, m/z]: 516.0 [M+H], 538.0 [M+Na]. HPLC (method 1): retention time = 24.77 min.

Biological assays

***In vitro* 2D monolayer assay**

This study was performed by CRO oncotest.

Tumor Cell Lines

The cell line panel used for assessing the novel inhibitors comprised four cell lines derived from human prostate cancers: LNCaP, 22Rv1, VCaP, and DU145. Cell lines were routinely passaged once or twice weekly and maintained in culture for up to 20 passages. All cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in RPMI 1640

medium (25 mM HEPES, with L-glutamine, #FG1385, Biochrom, Berlin, Germany) supplemented with 10% (v/v) foetal calf serum (Sigma, Taufkirchen, Germany) and 0.1 mg/mL gentamicin (Life Technologies, Karlsruhe, Germany).

Compounds Handling

Stock solutions for all inhibitors were prepared in DMSO at a concentration of 33 mM and aliquots of 200 µl were finally stored at -20°C. All compounds were well soluble at this concentration in DMSO and precipitation was not observed for any compound dissolved in 100% DMSO. Aliquots of the stock solution were thawed on the day of use and stored at room temperature prior to and during treatment. The subsequent dilutions were done with complete RPMI1640 cell culture medium. The DMSO stock solution was first diluted 1:22 (corresponding to 4.5% v/v DMSO). Starting with this solution, serial dilutions in half-log steps with cell culture medium were done using an intermediate dilution plate. Finally, 10 µl taken from the intermediate dilution plate were transferred to 140 µl / well of the cell culture plate. Thus, at the highest test concentration the DMSO stock was diluted 1:330, corresponding to a maximum DMSO concentration of 0.3 % v/v in the assay.

2D Cell Proliferation Assay

A modified propidium iodide (PI) based monolayer assay[81] was used to assess the anti-cancer activity of the compounds. Briefly, cells were harvested from exponential phase cultures, counted and plated in 96-well flat-bottom microtiter plates at a cell density of 4,000 – 20,000 cells/well depending on the cell line's growth rate. After a 24 h recovery period to allow the cells to resume exponential growth, 10 µl of culture medium (six control

wells/plate) or culture medium with test compound were added. The compounds were applied in half-log increments at 10 concentrations (0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.2, 10, 32, and 100 µM) in triplicate. After a total treatment period of 96 h, cells were washed with 200 µl PBS to remove dead cells and debris. Then, 200 µl of a solution containing 7 µg/ml propidium iodide (PI) and 0.1% (v/v) Triton X-100 was added. After an incubation period of 1-2 hours at room temperature, fluorescence (FU) was measured using the EnSpire Multimode Plate Reader (excitation λ= 530 nm, emission λ= 620 nm) to quantify the amount of attached viable cells. IC_{50} values were calculated by 4 parameter non-linear curve fit using Oncotest Warehouse Software. For calculation of mean IC_{50} values the geometric mean was used.

Androgen receptor agonist/antagonist assays

The AR assay was performed by Invitrogen™ Life Technologies.

The SelectScreen® Cell-Based Nuclear Receptor Profiling Service uses the GeneBLAzer® Betalactamase reporter technology. The GeneBLAzer® Validated Assays in the service are tested and documented to show a high level of performance.

Compounds Handling

Test compounds were received by Cyprotex as a 10 µM solution, in 100% DMSO. The 10 µM test compounds were serially diluted (10 point, 1/2-log increments) in 100% DMSO.

Full Stim Control.

The full stim control contained 0.1% DMSO, cells and a maximum concentration of the known stim (agonist, R1881). In agonist mode, the full stim control is used to determine the upper end of the assay or 100% activation. In antagonist mode, the full stim control was used to determine the actual EC_{80} used in the assay, with the EC_{80} concentration chosen from previous agonist experiments. The control was run on each plate for each cell line.

AR-Agonist Screen.

AR-UAS-bla GripTite™ 293 cells were thawed and re-suspended in Assay Media (DMEM phenol red free, 2% CD-treated FBS, 0.1 mM NEAA, 1 mM Sodium Pyruvate, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 312,500 cells/mL. 4 µL of a 10 µM of R1881 (control agonist, EC_{50} = 0.322 nM) or the compounds under investigation (10 µM concentration) were added to appropriate wells of a 384-well Poly-D-Lysine assay plate. 32 µL of cell suspension (10,000 cells) was added to each well. 4 µL of assay media was added to all wells to bring the final assay volume to 40 µL. The plate was incubated for 16-24 hours at 37 ºC /5% CO_2 in a humidified incubator. 8 µL of 1 µM substrate loading solution was added to each well and the plate incubated for 2 hours at room temperature. Fluorescence was then measured on a plate reader.

AR-Antagonist Screen

Activated by R1881: AR-UAS-bla GripTite™ 293 cells were thawed and prepared as described above for the Agonist Screen. 4 µL of a 10X serial dilution of Cyproterone Acetate (control antagonist starting concentration, 3.160 nM) or compounds under

investigation were added to appropriate wells of a Poly-D-Lysine assay plate. 32 µL of cell suspension was added to the wells and pre-incubated at 37ºC/5% CO_2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist R1881 at the pre-determined EC_{80} concentration was added to wells containing the control antagonist or compounds. The plate was incubated for 16-24 hours at 37ºC/5% CO_2 in a humidified incubator. 8 µL of 1 µM of substrate loading solution was added to each well and the plate incubated for 2 hours at room temperature. Fluorescence was then measured on a plate reader.

In vitro metabolic stability, plasma protein binding and cardiotoxicity studies

All *in vitro* biological evaluations were performed by CYPROTEX.

Microsomal Metabolic Stability

Microsomes (final protein concentration 0.5 mg/mL), 0.1 M phosphate buffer pH 7.4 and test compound (final substrate concentration 3 µM; final DMSO concentration 0.25 %) were pre-incubated at 37 °C prior to the addition of NADPH (final concentration 1 mM) to initiate the reaction. The final incubation volume is 50 µL. A control incubation was included for each compound tested where 0.1 M phosphate buffer pH 7.4 was added instead of NADPH (minus NADPH). Two control compounds Dextromethorphan and Verapamil were included with each species. All incubations were performed singularly for each test compound. Each compound was incubated for 0, 5, 15, 30 and 45 min. The control (minus NADPH) was incubated for 45 min only. The reactions were then stopped by transferring 20 µL of incubate to 60 µL methanol at the appropriate time points. The

incubation plates were centrifuged at 2,500 rpm for 20 min at 4 °C to precipitate the protein, the sample supernatants were combined in cassettes of up to 4 compounds and samples analyzed by LC-MS/MS.

Plasma protein binding

Test compound solutions (final concentration 5 µM) were prepared in both 100 % species-specific plasma and buffer (pH 7.4). The plasma solution was added to one side of the membrane in an equilibrium dialysis system while the buffer solution was added to the other side. The system was allowed to reach equilibrium at 37 °C. Compound concentration on both sides of the membrane was measured by LC-MS/MS and the fraction of unbound compound was calculated. The extent of binding is reported as % mean bounded.

hERG Channel Inhibition (IC_{50} Determination)

Chinese hamster ovary (CHO) cells (cell-line obtained from Cytomyx, UK) expressing the hERG potassium channel were dispensed into 384-well planar arrays and hERG tail-currents measured by whole-cell voltage-clamping. The experiments were performed on an IonWorksTM HT instrument (Molecular Devices Corporation), which automatically performs electrophysiology measurements in 48 single cells simultaneously in a specialised 384-well plate (PatchPlateTM). All cell suspensions, buffers and test compound solutions were at room temperature during the experiment. A single-cell suspension was prepared in extracellular solution (Dulbecco's phosphate buffered saline

with calcium and magnesium pH 7 - 7.2) and aliquots added automatically to each well of a PatchPlateTM. The resistance of each seal was measured via a common ground-electrode in the intracellular compartment and individual electrodes placed into each of the upper wells.

Electrical access to the cells was then achieved by circulating a perforating agent, amphotericin, underneath the PatchPlateTM and then measuring the pre-compound hERG current. An electrode was positioned in the extracellular compartment and a holding potential of -80 mV applied for 15 sec. The hERG channels were then activated by applying a depolarising step to +40 mV for 5 sec and then clamped at -50 mV for 4 sec to elicit the hERG tail current, before returning to -80 mV for 0.3 s. Test compound solutions (prepared by diluting 10 mM DMSO solutions into extracellular buffer to a final concentration of 25 μM, final DMSO concentration 0.25 %), were then added automatically to the upper wells of the PatchPlateTM from a 96-well microtitre plate. The test compounds were left in contact with the cells for 300 sec before recording currents using the same voltage-step protocol as in the pre-compound scan. Quinidine, an established hERG inhibitor, is included as a positive control and buffer containing 0.25 % DMSO is included as a negative control. Each concentration is tested in 4 replicate wells on the PatchPlateTM.

In vivo efficacy assay.

Male immunodeficient NMRI nu/nu mice from Harlan were used. The animals were delivered at the age of four to six weeks and used for implantation after at least one week of quarantine. All experiments are approved by the local authorities and are conducted

according to the guidelines of the German Animal Welfare Act (Tierschutzgesetz). For antitumor activity experiments mice were injected in the hind flank with tissue culture 22Rv1 cells patient derived. Animals and tumour implants were monitored daily until the maximum number of implants show clear signs of beginning solid tumour growth. When the tumor mass had reached 80 – 200 mm^3 the mice were randomized into groups and therapy with the selected inhibitor and docetaxel were performed for 4 weeks. Treatment with the investigational compound was given as 16-18 daily intraperitoneal (i.p.) doses, whereas docetaxel was given as three weekly intravenous (i.v.) treatments.The antitumoral efficacy of **48a** was then evaluated. Vehicle for **48a** and control vehicle was 10% Ethanol 10% Cremophor EL 5% dextrose. Control mice were treated with vehicle for the same treatment period. The absolute tumor volumes (ATVs) were determined by two-dimensional measurement with a caliper on the day of randomization and then twice weekly. Tumor volumes are calculated according to the formula: Tumor volume = (a x b^2) x 0.5 where a represents the largest and b the perpendicular tumor diameter of the tumor representing an idealized ellipsoid. Statistical significance of anti-tumor efficacy was evaluated on the days where the minimum T/C values are reached in the relevant test groups (day 14). Statistical analysis was only carried out if at least 50% of the initially randomized animals in a relevant group are still alive.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website. Experimental procedure and characterization data for all compounds synthesised;

Complete table of the antiproliferative activity for all compounds synthesized; Copy of the ^{1}H, ^{13}C, ^{19}F NMR spectra and HPLC traces for the most relevant compounds (**41b, 44n**, **45n**, **48a**, **48b**, **67a**, **79**, **83**).

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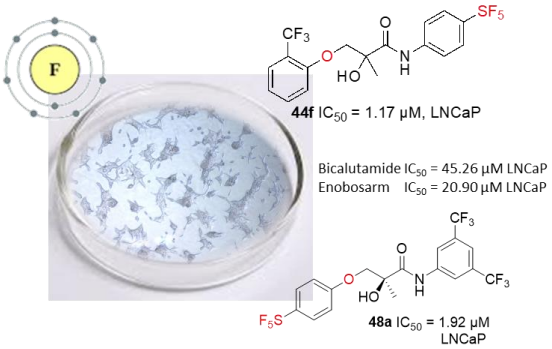
Table of Contents graphic


F
CF3
O
HO
N
H
SF5
44f IC50 = 1.17 µM, LNCaP
Bicalutamide IC50 = 45.26 µM LNCaP
Enobosarm IC50 = 20.90 µM LNCaP
CF3
O
HO
N
H
CF3
F5S
48a IC50 = 1.92 µM
LNCaP