Investigation of the Effects of Adenosine and Purine Nucleoside Analogues on the Viability of Human Breast Cancer Cells

A thesis in accordance with the conditions governing candidates for the degree of

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In Cardiff University

Presented by

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Publications

Henson AD, Simons C, Hutcheson IR and Kidd EJ. Anti-proliferative effects of cladribine on human breast MCF-7 adenocarcinoma cells. Proceedings of the British Pharmacological Society at http://www.pA2online.org/abstracts/Vol6Issue4abst 147P.pdf.

Henson AD, Simons C, Hutcheson IR and Kidd EJ. Differential effects of clofarabine on breast cancer cell proliferation. Proceedings of the British Association for Cancer Research 50th Anniversary Meeting. (In press).

Henson AD, Hutcheson IR, Simons C and Kidd EJ. (2010). Differential Effects of Clofarabine on Oestrogen Receptor-Positive and Negative Breast Cancer Cell Lines. (Manuscript in preparation).

Henson AD, Hutcheson IR, Simons C and Kidd EJ. (2010). Anti-Proliferative Effects of Adenosine and 2'-Deoxyadenosine on Human Breast Cancer Cell Lines. (Manuscript in preparation).

Summary

Breast cancer is the second leading cause of cancer death in women in the UK and so there is a need to develop new treatments for this. The aims of these studies were to investigate the effects of adenosine receptor agonists and antagonists, the endogenous compounds adenosine and 2'-deoxyadenosine and the clinically used purine nucleoside analogues on the viability of breast cancer cells *in vitro*.

Initial studies identified the expression of all four adenosine receptors in the MCF-7 (ER-positive) and MDAMB231 (ER-negative) breast cancer cell lines but these receptors were not involved *per se* in maintaining cell viability. Adenosine and 2'-deoxyadenosine were able to reduce cell viability by mechanisms involving adenosine receptor activation in the MCF-7 cells, a reduction in extracellular regulated kinase 1/2 (ERK 1/2) phosphorylation in the MDAMB231 cells and intracellular phosphorylation by adenosine kinase in both cell lines.

Further studies identified that the clinically used 2'-deoxyadenosine analogue, clofarabine, had equal potency in the ER-negative cell lines compared to a leukaemia cell line *in vitro*. The ER-positive cells were 8-fold more resistant to clofarabine, however. Inhibition of ribonucleotide reductase appeared to be the primary mechanism of action of clofarabine and cladribine, but not fludarabine in the MCF-7 and MDAMB231 cell lines. ERK 1/2 signalling was also partly required for the action of clofarabine in the MDAMB231, but not the MCF-7 cells.

Studies on the resistance of clofarabine in ER-positive cells identified that increased basal expression of the metabolising enzyme, cytostolic 5'-nucleotidase II and the ribonucleotide reductase subunit, p53R2, were correlated with decreased sensitivity to clofarabine. Drug-induced p53R2 expression in p53 wild-type MCF-7 cells also contributed to resistance.

The present study highlights the potential for clofarabine as a treatment for breast cancers, particularly ER-negative cancers where patients have a very poor prognosis.

Contents

Abbreviations

ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AMP	Adenosine monophosphate
APAF 1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
AMPK	Adenosine monophosphate-activated protein kinase
cAMP	Cyclic adenosine monophosphate
cN-II	Cytostolic 5'-nucleotidase II
ClofTP	Clofarabine triphosphate
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
CREB	cAMP response element binding protein
Ct	Crossing threshold
dAMP	Deoxyadenosine monophosphate
dADP	Deoxyadenosine diphosphate
dATP	Deoxyadenosine triphosphate
dCF	Deoxycoformycin
dCK	Deoxycytidine kinase
dFdCTP	Gemcitabine triphosphate
dNTP	Deoxynucleotide triphosphate
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
ENT	Equilibrative nucleoside transporter
ER	Oestrogen receptor
ERK 1/2	Extracellular regulate kinase 1 and 2
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
GAPDH	Glyceralderhyde-3-phosphate dehydrogenase
GOI	Genes of interest
GPCR	G-protein coupled receptors
Her2	Epidermal growth factor receptor 2
HPLC	High performance liquid chromatography
IAP-2	Inhibitor of apoptosis protein 2
IB-MECA	1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide
IBC	Inflammatory breast cancer
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
JNK	c-Jun N-terminal kinases
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
NA	Nucleoside analogue
NAD^+	Nicotinamide adenine dinucleotide

NBMPR	Nitrobenzylmercaptopurine riboside
NDPK	Nucleoside diphosphate kinase
NECA	5'-*N*-Ethylcarboxamidoadenosine
NMPK	Nucleoside monophosphate kinase
NPI	Nottingham Prognostic Index
NT	Nucleoside transporter
p53R2	p53-induced ribonucleotide reductase subunit 2
PARP	Poly(ADP-ribose) polymerase
PI	Propidium iodide
PI3K	Phosphatidylinositol-3'-kinase
PNA	Purine nucleoside analogue
PR	Progesterone receptor
REST	Relative expression software tool
RRM1	Ribonucleotide reductase subunit 1
RRM2	Ribonucleotide reductase subunit 2
RT-PCR	Real Time-Polymerase Chain Reaction
SCID	Severe combined immunodeficiency disorder
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SDS	Sodium dodecyl-sulfate
SERM	Selective oestrogen receptor modulators
TNBC	Triple negative breast cancer
TNFα	Tumour necrosis factor alpha
TNM	Tumour, Nodal, Metastasis (TNM Classification of Malignant Tumours)
VEGF	Vascular endothelial growth factor

Chapter 1: General Introduction

1.1 Adenosine and 2'-Deoxyadenosine

1.1.1 Structure and Physiological Roles

Adenosine (9-β-D-ribofuranosyladenine) is a purine nucleoside with a structure consisting of a 6-aminopurine (adenine) joined to a ribose (ribofuranose) moiety by a β-glycosidic bond. Adenosine is found both intra- and extracellularly and also exists as 2'-deoxyadenosine with the deletion of the hydroxyl at the C2 position on the ribose (Figure 1.1).

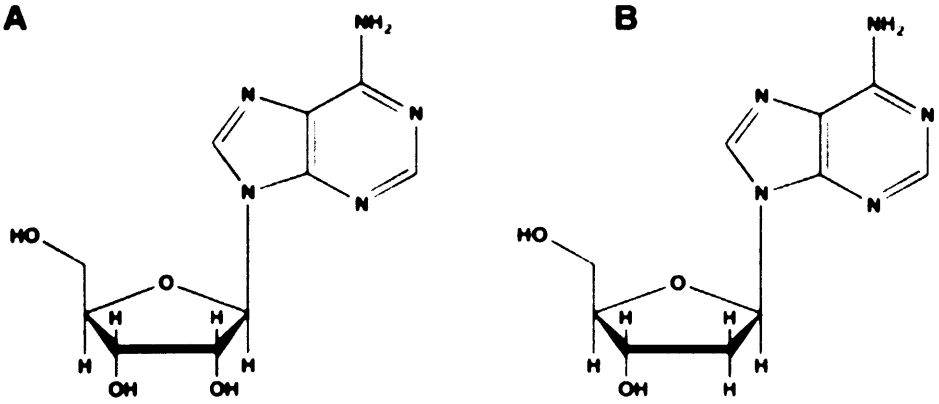


Figure 1.1 Structure of Adenosine (A) and 2'-Deoxyadenosine (B)

Adenosine serves a variety of physiological roles, particularly as an autocrine and paracrine signalling and regulatory molecule (Borowiec *et al.* 2006). The regulatory functions of adenosine include action as an anti-inflammatory agent (Cronstein 1994), regulator of blood flow and modulator of neuronal excitability in the central nervous system (CNS; Davis *et al.* 2003) with functions to inhibit the release of neuronal transmitters (Yoon and Rothman 1991). During ischaemia or inflammation, extracellular adenosine accumulates past normal physiological levels to aid in tissue protection and regeneration through ischaemic post-conditioning, triggering anti-inflammatory responses and stimulation of angiogenesis (Linden 2005). 2'-Deoxyadenosine and other deoxypurines and pyrimidines form the nucleobases of DNA.

1.1.2 Synthesis

Adenosine is continually produced and metabolised and can be synthesised both intra- and extracellularly via two distinct pathways (Figure 1.2) (Fredholm *et al.* 2001). The primary pathway involves dephosphorylation of adenine nucleotides by intracellular and extracellular 5'-nucleotidases (Madrid-Marina and Fox 1986; Löffler *et al.* 2007). The second pathway involves demethylation of *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH) and subsequent hydrolysis of SAH by SAH hydrolase to adenosine and homocysteine (Luippold *et al.* 1999), although this process only occurs intracellularly. Synthesis of 2'-deoxyadenosine occurs via reduction of adenosine diphosphate (ADP) to deoxyadenosine diphosphate (dADP) and then subsequent dephosphorylation by nucleotidase enzymes.

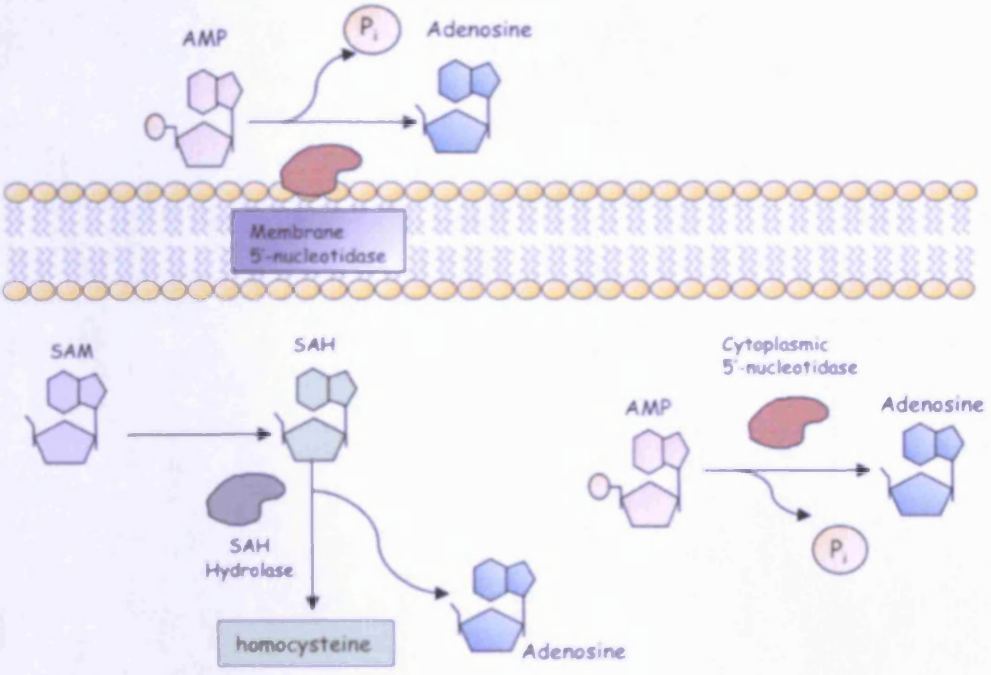


Figure 1.2 Synthesis of Adenosine

Adenosine is synthesised intra- and extracellularly by dephosphorylation of AMP and by hydrolysis of SAH to homocysteine and adenosine.

1.1.3 Metabolism

When levels of adenosine or 2'-deoxyadenosine exceed normal physiological conditions, they are rapidly deaminated by adenosine deaminase (ADA) to inosine or phosphorylated to AMP by adenosine kinase (Figure 1.3) (Lloyd and Fredholm 1995; Deussen *et al.* 1999). Whilst ADA works mainly intracellularly, it also functions extracellularly by being bound to the cell membrane, anchored by membrane proteins CD26 and A_1 and A_{2B} adenosine receptors (Romanowska *et al.* 2007).

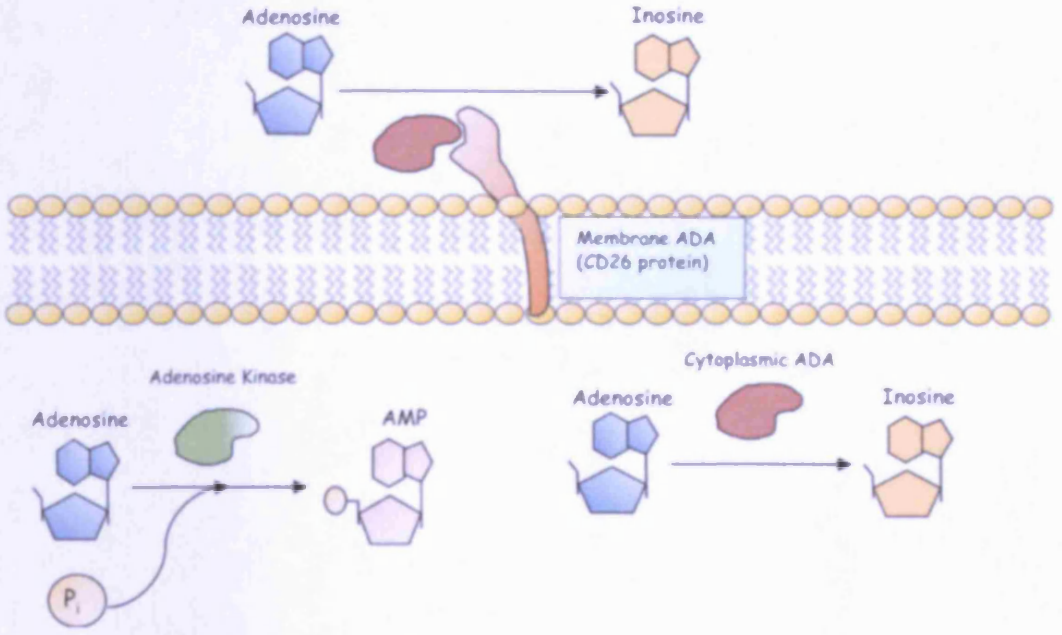


Figure 1.3 Metabolism of Adenosine

Adenosine is deaminated by ADA both intra- and extracellularly to inosine and is also phosphorylated by adenosine kinase to AMP.

1.1.4 Nucleoside Transporters

Nucleoside transporters (NTs) play important roles in physiological functioning as nucleosides are highly hydrophilic and require active transport to cross cellular membranes (Löffler *et al.* 2007). NTs also play an important role in anti-cancer and anti-viral chemotherapy where they are involved in transportation of nucleoside analogues (NAs) across the plasma membrane (Fang *et al.* 1996).

There are two classes of NTs expressed on mammalian cells. The first are the equilibrative nucleoside transporters (ENT), which are passive transporters working on the concentration gradient of nucleosides. ENTs are more widely distributed than concentrative nucleoside transporters (CNTs) (Baldwin *et al.* 1999) and thus play a greater role in adenosine homeostasis. There are four subclasses of ENTs, 1-4, with 1 and 2 being the most well studied isoforms, and both selectively transport adenosine and other nucleosides (Damaraju *et al.* 2003). ENT 1 and 2 are distinguished based on their sensitivity to the NT inhibitor, nitrobenzylmercaptopurine riboside (NBMPR), with ENT1 being NBMPR-sensitive and ENT2 being NBMPR-insensitive (Damaraju *et al.* 2003; Löffler *et al.* 2007; Zhang *et al.* 2007).

The second class is the CNTs which are sodium-dependent active transporters that transport against the concentration gradient (Fang *et al.* 1996; Löffler *et al.* 2007). There are 3 subsets of CNTs classified on their substrate selectivity; CNT1 which predominantly transports pyrimidine nucleosides; CNT2 that favours purine substrates and CNT3 that has broad substrate specificity and transports purines and pyrimidines (Baldwin *et al.* 1999; Archer *et al.* 2004).

1.2 Adenosine Receptors

1.2.1 Structure, Classification and Distribution

Purinergic surface receptors were first identified in 1976 and were later sub-divided into those with affinity for adenosine, and those with affinity for adenine nucleotides deemed P_1 and P_2, respectively (Burnstock 2006). The physiological actions of adenosine are mediated by P_1 specific adenosine receptors termed A_1, A_{2A}, A_{2B} and A_3 and each receptor has a distinct pharmacological profile (Klinger *et al.* 2002). The wide distribution of adenosine receptors in different cells and tissues means that adenosine can influence nearly every tissue (Klinger *et al.*

2002; Chang *et al.* 2004) with cells almost always expressing multiple adenosine receptor subtypes (Fredholm *et al.* 2001).

Adenosine receptors are G protein-coupled receptors (GPCRs) (Fredholm *et al.* 2001; Klinger *et al.* 2002; Jacobson and Gao 2006). In humans the A_{2A} and A_{2B} receptors have the most homology at 59%, while the human A_1 and A_3 receptors share 49% homology (Jacobson and Gao 2006) These receptors show the typical topological structure common to all GPCRs, with seven α-helical membrane-spanning domains with an extracellular amino terminus and an intracellular carboxyl terminus (Olah and Stiles 2000). Previous work on GPCRs suggested that it is the binding of a receptor agonist to the extracellular domain that affects orientation of the transmembrane domains of the receptor, which in turn causes conformational changes in the intracellular aspect of the receptor that can elicit G protein activation and thus physiological responses (Olah and Stiles 2000).

1.2.2 Adenosine Receptor Signalling

Adenosine receptors initiate their intracellular signals through G protein coupling. Classically, it was thought that the intracellular signalling pathways associated with adenosine receptors involved either activation or inhibition of adenylate cyclase regulating cyclic adenosine monophosphate (cAMP) levels. However, receptors have also been associated with phospholipase C, Ca^{2+} and mitogen-activated protein kinase (MAPK) pathways (Jacobson and Gao 2006). As a basic overview, both A_1 and A_3 receptors act as inhibitory receptors, mediating decreases in cAMP by G_i and G_o proteins. A_{2A} and A_{2B} receptors, however, act as stimulatory receptors, increasing cAMP levels by G_s proteins (Klinger *et al.* 2002; Lynge *et al.* 2003; Schulte and Fredholm 2003).

The cAMP-dependent pathway involves activation of protein kinase A, which can phosphorylate a multitude of other proteins and transcription

factors, such as cAMP response element binding protein (CREB) (Daniel *et al.* 1998). CREB is well known for its role in cell proliferation and switches on several genes associated with oncogenesis such as *c-fos* and inactivating tumour suppressor genes (Siu and Jin 2007). Nishihara *et al.* (2004) found that activation of the cAMP pathway inhibited apoptosis in colon cancer cells by phosphorylation of CREB and induced activation of inhibitor of apoptosis protein 2 (IAP-2), via the extracellular regulated kinases 1 and 2 (ERK 1/2) and p38 MAPK pathways.

Adenosine receptors are also linked to activation of the phospholipase C/inositol triphosphate/diacylgyclerol/protein kinase C pathway, where it acts on cell growth, differentiation, apoptosis and intracellular signalling mechanisms (Brandt *et al.* 2002). Activation of the phosphatidylinositol-3'-kinase (PI3K)-dependent protein kinase B (Akt) pathway by adenosine receptors is known to inhibit apoptosis via regulation of transcription factors such as Bcl-2, apoptotic inducers and cell cycle regulators (Vanhaesenbroeck and Alessi 2000; Downward 2004; Hanada *et al.* 2004).

Adenosine has also been shown to influence ion channel activation via adenosine receptor stimulation including K_{ATP} channels and release of intracellular Ca^{2+} that help in cell protection from ischaemia (Mogul *et al.* 1993; Tracey *et al.* 1998; Haas and Selbach 2000; Andoh *et al.* 2006).

MAPKs are a well-conserved group of proline-directed serine/threonine kinases that transduce extracellular signals with control over a variety of cellular responses that include cell growth, division, differentiation and apoptosis (Chen *et al.* 2001; Luttrell 2003; Shulte and Fredholm 2003). Over 20 MAPKs have been discovered in mammals (Chen *et al.* 2001), with three primary subfamilies: p42/p44 ERK 1/2, c-Jun N-terminal kinases (JNK) and the p38 MAPK (Robinson and Dickenson 2001).

The ERK/MAPK pathway is one of the most well studied signal transduction pathways. This pathway is amongst the most important for control of cell cycle proliferation and is known to activate various proto-oncogenes (Fang and Richardson 2005). The ERK 1/2 pathway is known to be activated in both naïve and transfected cell lines by A_1 (Dickenson *et al.* 1998; Germack and Dickenson 2000; Robinson and Dickenson 2001; Brust *et al.* 2006; Lee *et al.* 2007), A_{2A} (Sexl *et al.* 1997; Seidel *et al.* 1999; Merighi *et al.* 2002; Rivo *et al.* 2007), A_{2B} (Feokistov *et al.* 1999; Gao *et al.* 1999; Schulte and Fredholm 2000, 2002 and 2003; Grant *et al.* 2001) and A_3 receptors (Neary *et al.* 1998; Graham *et al.* 2001; Schulte and Fredholm 2000, 2002; Trincavelli *et al.* 2002; Hammarberg *et al.* 2003).

1.3 Adenosine and Cancer

1.3.1 Adenosine Receptor Expression in Tumours

The expression of adenosine receptors in tumours has been relatively well studied. However, varying results have been found in breast cancer cell lines. Lu *et al.* (2003) and Cheung *et al.* (2006) found that MCF-7 cells do not express mRNA for the A_3 adenosine receptor, however, Panjehpour and Karami-Tehrani (2004) found that MCF-7 cells did express A_3 adenosine receptor mRNA, but at very low levels. Cheung *et al.* (2006) then found that MCF-7 cells expressed the mRNAs for the A_{2A} and A_{2B} receptors only, yet Panjehpour *et al.* (2005) noted that MCF-7 cells were devoid of adenosine receptor expression. This group then identified MCF-7 and MDA-MB468 cells that express mRNA for all adenosine receptors (Panjehpour and Karami-Tehrani 2007). These data highlight clear inconsistencies in the expression of adenosine receptors that may be due to experimental methods or variation amongst the cell lines. Recent works have also identified that A_1 receptor expression is regulated by the oestrogen receptor alpha (ERα) in ER-positive MCF-7 cells (Lin *et al.* 2010).

The expression of A_1 receptors have been reported in colorectal (Khoo *et al.* 1996; Gessi *et al.* 2007), melanoma (Merighi *et al.* 2003) and leukaemia cells (Gessi *et al.* 2001). A_{2A} receptors are expressed on epidermoid (Tey *et al.* 1992), leukaemia (Gessi *et al.* 2001; Munro *et al.* 1998), lymphoma (Munro *et al.* 1998), colorectal (Gessi *et al.* 2007) and melanoma (Merighi *et al.* 2003) cell lines. A_{2B} receptors have also been found on colorectal (Gessi *et al.* 2007), leukaemia (Gessi *et al.* 2001) and melanoma cell lines (Merighi *et al.* 2003). The A_3 receptor has been identified on melanoma (Merighi *et al.* 2003; Fishman *et al.* 2002), colorectal (Gessi *et al.* 2007) and leukaemia cells (Kohno *et al.* 1996; Gessi *et al.* 2001).

1.3.2 Stimulatory Effects of Adenosine on Tumours

Adenosine and 2'-deoxyadenosine are normal physiological metabolites with a diverse range of functions. During hypoxia adenosine levels rise in the extracellular matrix to 5–40 μM, depending upon tissue type and the basal concentration of adenosine, which is usually between 50 and 500 nM (Schubert *et al.* 1994; Blay *et al.* 1997; Harrison *et al.* 1998). This suggests that adenosine has a role in cellular protection during ischemia. Indeed, it has been demonstrated that adenosine and selective agonists can activate A_{2A} receptors and A_{2B} receptors to induce the expression of vascular endothelial growth factor (VEGF) and downregulate the anti-angiogenic matrix protein, thrombospondin I, which stimulates angiogenesis (Grant *et al.* 2001; Feoktistov *et al.* 2002; Leibovich *et al.* 2002; Gardener and Olah 2003; Montesinos *et al.* 2004; Desai *et al.* 2005). As solid tumours are frequently exposed to hypoxia until they can develop a blood supply, this strongly suggests that adenosine is tumour promoting *in vivo* and has been suggested as the mechanism by which tumours acquire the necessary vasculature (Ferrara 2002).

Adenosine is also known to promote tumour growth by other mechanisms. Adenosine is known to modulate immune function, and

levels have been demonstrated to rise in the extracellular fluid to concentrations that are immunosuppressive (Blay *et al.* 1997). Adenosine has also been shown to inhibit T-killer cell activation and adhesion to carcinoma cells (Hoskin *et al.* 1994; MacKenzie *et al.* 1994) and suppress production of interleukin-12 and tumour necrosis factor alpha (TNFα) (Haskó *et al.* 2000). Similarly, Ohta *et al.* (2006) found that adenosine-stimulated A_{2A} receptor activation on T cells inhibited their action against mouse solid tumours.

Adenosine has also been shown to activate adenosine receptors on cancer cells directly to promote tumour cell survival. Activation of the A_1 receptor by adenosine was able to induce chemotaxis in A2058 human melanoma cells (Woodhouse *et al.* 1998). Several studies have also shown that activation of A_1 and A_3 receptors can protect against chemotherapy-induced apoptosis (Fishman *et al.* 2000; Gao *et al.* 2001; Shneyvays *et al.* 2001) and ischaemic injury (Safran *et al.* 2000). Gessi *et al.* (2007) found that adenosine-stimulated A_3 receptor activation increased the proliferation of colon cancer cell lines and addition of ADA inhibited their proliferation. Mujoomdar *et al.* (2004) also showed that adenosine had mitogenic effects on the breast cancer cell lines MCF-7 and T47D and colon cancer cell lines between 10-100 μM.

1.3.3 Inhibitory Effects of Adenosine on Tumours

Despite evidence that adenosine can promote tumour survival, there are data that show that adenosine and 2'-deoxyadenosine can inhibit the proliferation of tumours. These effects have been attributed to both intracellular and adenosine receptor-mediated mechanisms.

1.3.3.1 Adenosine Acts by an Intracellular Mechanism

Adenosine and 2'-deoxyadneosine have been shown to inhibit proliferation or induce apoptosis in breast (Barry and Lind 2000; Hashemi *et al.* 2005), gastric (Saitoh *et al.* 2004), colon (Barry and Lind 2000) hepatoma (Wu *et al.* 2006; Yang *et al.* 2007), ovarian (Barry and

Lind 2000) and leukaemia (Tanaka *et al.* 1994; Schneider *et al.* 2001) cell lines. These studies suggested that adenosine and 2'-deoxyadenosine were phosphorylated by adenosine kinase to the monophosphates and subsequently to the triphosphates that disrupted deoxynucleotide triphosphate (dNTP) balance and caused DNA strand breakage.

This idea that intracellular phosphorylation is required for the activity of adenosine is further compounded by the clinically used 2'-deoxyadenosine analogues, cladribine, clofarabine and fludarabine, all having similar mechanisms of action that require intracellular phosphorylation to the mono- and subsequently triphosphates to become active (Månsson *et al.* 1999). Similarly, deoxycoformycin (pentostatin) is a potent and irreversible inhibitor of ADA, which causes accumulation of adenosine triphosphate (ATP) and deoxyadenosine triphosphate (dATP). These inhibit ribonucleotide reductase and cause imbalance of the dNTP pool, which in turn inhibits DNA synthesis and results in cell death (Weiss *et al.* 2003).

1.3.3.2 Extracellular Actions of Adenosine via Adenosine Receptors

Evidence suggests that modulation of adenosine receptors are able to inhibit the growth of a variety of cancer cell lines. Activation of the A_1 adenosine receptor has been shown to inhibit the growth of breast (Colquhoun and Newsholme 1997), epidermoid (Tey *et al.* 1992) and astrocytoma (Sai *et al.* 2006) cancer cells. Interestingly, this study by Sai *et al.* (2006) found that along with A_1-mediated mechanisms, adenosine could also cause apoptosis by intracellular mechanisms involving phosphorylation by adenosine kinase and activation of AMP-activated protein kinase (AMPK).

To date there is limited evidence to suggest the involvement of either A_{2A} or A_{2B} receptors in mediating growth inhibitory effects. A study by

Merighi *et al.* (2002) demonstrated that activation of the A_{2A} receptor had anti-proliferative effects in melanoma cells but higher concentrations of adenosine activated the A_3 receptor and increased cell proliferation.

The A_3 receptor is by far the most widely studied receptor in terms of having anti-cancer potential. Activation of the A_3 receptor by either the endogenous ligand, adenosine, or selective agonists has been shown to inhibit the growth of lymphoma (Fishman *et al.* 2000), leukaemia (Kohno *et al.* 1996; Yao *et al.* 1997; Kim *et al.* 2002; Lee *et al.* 2005), breast (Panjehpour and Karami-Tehrani 2004; 2007), lung (Kim *et al.* 2008), colon (Ohana *et al.* 2001) and hepatocellular (Bar-Yehuda *et al.* 2008) cancer cell lines.

1.3.3.3 Anti-Proliferative Effects by Other Mechanisms

Cheung *et al.* (2006) demonstrated that the A_3 selective agonist, LJ-529, inhibited the growth of breast cancer cell lines via a mechanism independent of adenosine receptors and intracellular phosphorylation. Apoptosis was induced by activation of caspase 3 and c-poly(ADP)ribose polymerase (PARP) and down-regulation of the Wnt signalling pathway and Her2 expression. Similarly to this, Lu *et al.* (2003) found that the A_3 receptor agonist, IB-MECA, inhibited the growth of ER-positive breast cancer cells by down-regulation of ERα.

Furthermore El-Darahali *et al.* (2005) suggested that adenosine was able to induce cell death in the mouse thyroma (EL-4) cell line via extracellular adenosine that was not linked to adenosine receptors, but suggested the presence of a non-classical adenosine receptor. This is supported by Apasov *et al.* (2000) who also suggested the expression of a non-classical adenosine receptor 'X' in mouse thymocytes.

1.4 Nucleoside Analogues

The purine or pyrimidine NAs are amongst the most common class of anti-cancer drugs that are used clinically. These drugs have modifications on the ribose moiety, the purine or pyrimidine base, or both that aid in their uptake, increase substrate binding and improve their ability to pass the natural degradation pathways of nucleosides. They mimic the endogenous purines and pyrimidines and act as false substrates.

1.4.1 Synthesis of Deoxyribonucleotides

Purine and pyrimidine deoxyribonucleotides are essential in the synthesis and repair of DNA. Their synthesis occurs via two distinct mechanisms, the *de novo* and salvage pathways (Figure 1.4). The *de novo* pathway is the primary pathway for synthesis of deoxyribonucleotides in normal, differentiated cells but in rapidly proliferating cells such as cancer cells, the salvage pathway becomes the predominant pathway (Hatse *et al.* 1999). The purine *de novo* pathway begins with the synthesis of inosine monophosphate (IMP) from the purine precursor, 5-phosphoribosyl-1-pyrophosphate (PRPP). IMP is then converted to succinyl-AMP by adenylosuccinate synthetase and then to AMP by AMP lyase (Hatse *et al.* 1999). AMP is phosphorylated to ADP and can then undergo reduction to dADP by ribonucleotide reductase via reduction of the hydroxyl on the ribose (Cerqueira *et al.* 2005). In the salvage pathway, dATP is formed by successive phosphorylation of 2'-deoxyadenosine, deoxyadenosine monophosphate (dAMP) and deoxyadenosine diphosphate (dADP) by nucleoside kinases.

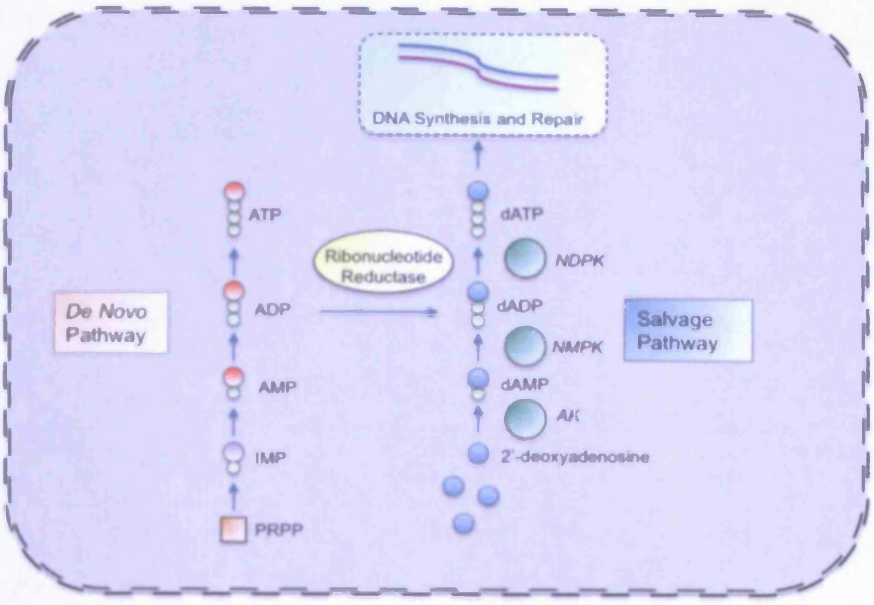


Figure 1.4 Synthesis of Purine Deoxynucleotides

In the de novo pathway, PRPP is converted to IMP, and then successively to AMP, ADP and ATP. ATP then goes on for use as an energy source or synthesis into the second messenger, cAMP. ADP can be reduced by ribonucleotide reductase to dADP, which is then phosphorylated to dATP for use in DNA synthesis and repair processes. In the salvage pathway, 2'-deoxyadenosine from nutrients and degraded DNA is successively phosphorylated by adenosine kinase (AK) to dAMP and then on to dADP and dATP by nucleoside mono(NMPK) and diphosphate kinases (NDPK), respectively.

To date, there are four clinically used adenosine analogues used in anti-cancer treatment, although there is current research being undertaken for their use in other conditions such as multiple sclerosis based on their immunosuppressive activity (Beutler *et al.* 1995; Leist and Vermarsch 2007). These drugs come under two categories; deoxycoformycin is the only inhibitor of ADA, whereas cladribine, fludarabine and clofarabine are halogenated nucleosides. Their structures are shown in Figure 1.5.

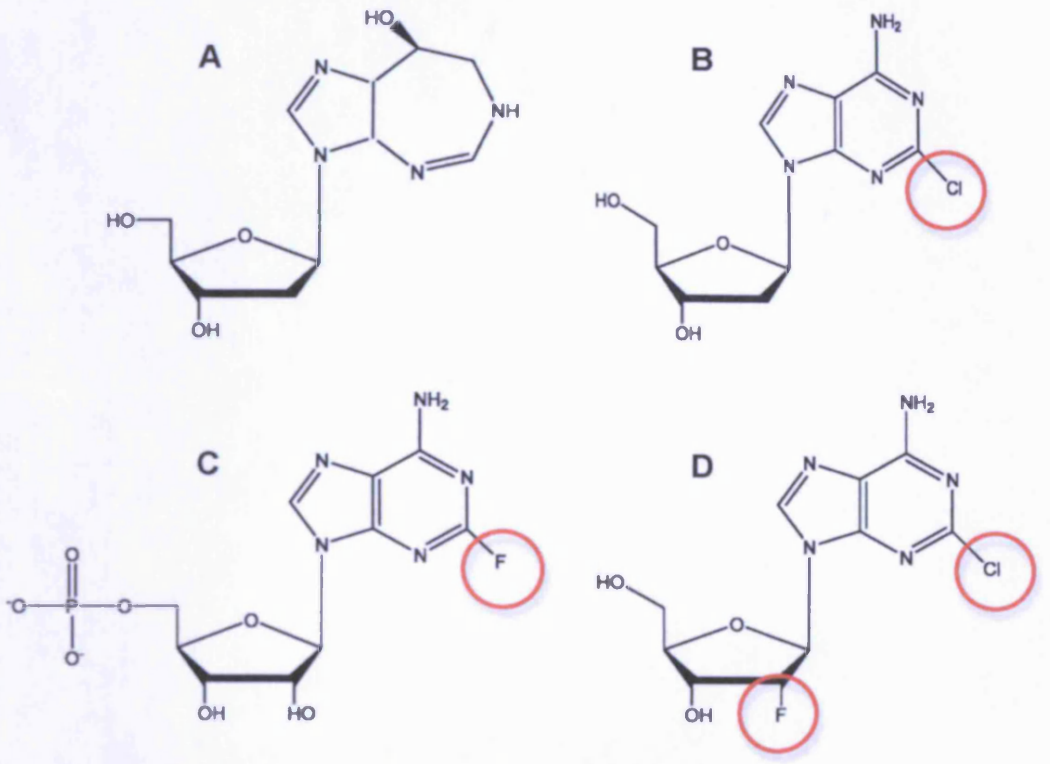


Figure 1.5 Clinically Used Nucleoside Analogue Structures. *Structures of deoxycoformycin (A), cladribine (B), fludarabine phosphate (C) and clofarabine (D). The different halogenations of cladribine, fludarabine phosphate and clofarabine are circled in red.*

1.4.2 Deoxycoformycin

Deoxycoformycin is the only ADA inhibitor used clinically and is also unique in that it is the only naturally occurring agent from this class, originally isolated from *Streptomyces antibioticus* (Woo *et al.* 1974). Originally, it was thought to be more active in T-cell based malignancies, due to the high levels of ADA in cells of T-cell origin, although this eventually proved to be incorrect, with cells of both B and T-cell origin proving to be susceptible to deoxycoformycin (Ho and Hensel 2006). Clinically it is used to treat hairy cell leukaemia, B-cell chronic lymphocytic leukaemia and indolent Non-Hodgkin's lymphomas including Waldenström's macroglobulinemia (Ho and Hensel 2006).

Molecularly, it mimics the transition state of adenosine to inosine via deamination by ADA. It has a hydroxyl group at the 4 position of the purine ring in place of an amino group (Figure 1.4 A). Although its exact mechanism(s) of action are still not fully understood, it is thought that inhibition of ADA results in an accumulation of 2'-deoxyadenosine that is converted to dATP by purine kinases, and this inhibits ribonucleotide reductase and SAH hydrolase resulting in decreased DNA synthesis and repair and eventual cell death (Fidias *et al.* 1996; Dillman 2004).

1.4.3 Fludarabine

Fludarabine is the most widely used purine nucleoside analogue (PNA) and is the gold standard for treatment of chronic lymphocytic leukaemia and Waldenström's macroglobulinaemia and is also used in conjunction with cytarabine to treat acute myeloid leukaemia (Fidias *et al.* 1996; Lenz *et al.* 2004; Robak and Robak 2007). Fludarabine was originally synthesised as the ADA resistant form of the anti-viral drug, vidarabine. Fludarabine is a halogenated adenosine analogue, but unlike cladribine or clofarabine, is given as the monophosphate form due to its relative insolubility as a nucleoside (Figure 1.4 B) (Fidias *et al.* 1996). Fludarabine is dephosphorylated before it enters the cell by membrane-bound 5'-nucleotidase, where it is then transported intracellularly via NTs and phosphorylated by deoxycytidine kinase (dCK) (Mackey *et al.* 1998; Galmarini *et al.* 2001). Fludarabine has similar mechanisms of action to other PNAs, whereby the triphosphate derivative has the most activity. Fludarabine triphosphate is an inhibitor of ribonucleotide reductase, which causes a reduction in dNTP pools, and subsequent DNA chain termination. The depletion of dATP and dCTP acts to self-potentiate fludarabine and other NAs. Depletion of dCTP increases cellular levels of dCK, thus increasing the phosphorylation of NAs. The reduced dATP levels mean that more of the NA drugs are then incorporated into the DNA (Galmarini *et al.* 2001). Fludarabine triphosphate is also an inhibitor of DNA polymerase-α, whereby it

competes with dATP for incorporation into the ATP sites of extending DNA chains and thus inhibits the polymerase. Also, once incorporated into the DNA chain, the ssDNA strand cannot be extended further at the 3'-end, causing DNA chain termination (Huang *et al.* 1990). Fludarabine has also been demonstrated to be an inhibitor of DNA ligase I which inhibits the ligation of AMP on Okazaki fragments during DNA synthesis or repair (Yang *et al.* 1992). Catapano *et al.* (1991) also found that fludarabine triphosphate was able to inhibit DNA primase and thus reduce the numbers of RNA primers available for continued DNA synthesis, and that this subsequently corresponded to impaired DNA synthesis or repair. Another mechanism of action for fludarabine is due to its incorporation into RNA and inhibition of RNA polymerase that results in inhibition of RNA transcription and ultimately leading to a decrease in the ability to produce proteins. (Huang and Plunkett 1991).

Similar to cladribine and deoxycoformycin, fludarabine has shown good activity against non-dividing cells most likely involving a mechanism of inhibition of the DNA repair process and activation of PARP (Pettitt *et al.* 2000).

1.4.4 Cladribine

Cladribine (2'-chloro-2'-deoxyadenosine) is a halogenated analogue of 2'-deoxyadenosine and was developed from findings that children with severe combined immunodeficiency disorder (SCID) had low or non-existent levels of ADA and subsequently elevated levels of dNTPs that caused lymphotoxicity (Saven and Piro 1994). Carson *et al.* (1980) evaluated a series of ADA-resistant deoxynucleotides, and found cladribine to be the most active and potent. Cladribine was first synthesised in 1972 by Christensen *et al.* (1972) but was only then looked at for its anti-lympho proliferative actions by Carson in 1980. Clinically, cladribine is currently used in the treatment of hairy cell leukaemia where it is most effective, but it is also used in other malignancies including chronic lymphocytic leukaemia, acute

myelogenous leukaemia, non-Hodgkin's lymphoma and Waldenström's macroglobulinaemia (Bryson *et al.* 1993). There has also been recent interest in using cladribine to treat multiple sclerosis based upon its immunotoxicity and it is currently undergoing clinical trials for this indication, with promising results so far (Beutler *et al.* 1995; Leist and Vermarsch 2007).

Structurally, cladribine is a 2'-deoxyadenosine analogue, which is halogenated at the 2' position on the purine ring with a chlorine atom (Figure 1.4 C). This halogenation confers resistance to deamination by ADA. Cladribine has well described mechanisms of action, similar to fludarabine and clofarabine. This class of drugs are known as prodrugs, as they require some degree of modification or chemical rearrangement once inside the cell or body, and are clinically inactive in their administered form.

The cytotoxic effects of cladribine require it to be transported intracellularly whereby it undergoes primary phosphorylation by nucleoside kinases such as dCK or deoxyguanosine kinase to the monophosphate, and is then successively phosphorylated to the di- and triphosphates. Intracellular cytostolic 5'-nucleotidase II (cN-II) is primarily responsible for the dephosphorylation of these 2'-chloro-nucleotides (Månsson *et al.* 1999), but cN-I can also dephosphorylate these nucleotides, albeit to a significantly lesser extent (Fyrberg *et al.* 2007). Cladribine triphosphate is the primary cytotoxic metabolite of cladribine, and inhibits various enzymes responsible for DNA replication and repair including ribonucleotide reductase and DNA polymerase (Griffig *et al.* 1989; Bontemps *et al.* 2000). As the triphosphate it is incorporated into the DNA template, which results in inhibition of DNA polymerase as it cannot elongate the DNA past this analogue, with the end result being DNA strand breakage and activation of the apoptotic cascade (Plunkett and Saunders 1991).

Cladribine has also been shown to induce alterations to mitochondrial function and integrity, factors that may be another cause of cladribine's cytotoxicity (Pérez-Galán 2002). Cladribine has been shown to be incorporated into mitochondrial DNA by mitochondrial DNA polymerase, and this can lead to inhibition of synthesis of mitochondrial proteins, that can disrupt the electron transport chain and oxidative phosphorylation (Galmarini *et al.* 2001). Similarly Marzo *et al.* (2001) demonstrated that caspase activation and cell death was preceded by a loss in mitochondrial transmembrane potential after exposure to cladribine.

1.4.5 Clofarabine

Clofarabine (2-chloro-2'-deoxy-2'-fluoro-β-D-adenosine) is the latest generation of PNAs and is again similar to fludarabine and cladribine in terms of structure and mechanisms of action. Clofarabine was discovered in a program set up in the 1980's and 90's to identify more novel NAs as anti-cancer agents. Both cladribine and fludarabine were in clinical trials but it was noted that both were susceptible to β-glycosidic bond cleavage between the ribose and the purine ring, either enzymatic and hydrolytic with cladribine (Lindemalm *et al.* 2004) or by phosphorylases with fludarabine (Bonate *et al.* 2006). Substitutions at the C2' position either with a hydroxyl group or other substitutes resulted in stability of the glycosidic bond, and it was eventually identified that a fluorine substitution conferred most stability. A chlorine substitution at the C2' position on the purine ring conferred resistance to ADA and this confirmation had the most anti-cancer activity (Figure 1.4 D) (Montgomery and Secrist 1997). Unlike fludarabine and cladribine, clofarabine is a poor substrate for purine nucleoside phosphorylase, the enzyme responsible for the glycosidic bond cleavage of adenine and ribose moieties. This combined with the 2'-fluoro substitution which resulted in increased acid stability, makes clofarabine more stable and increases its oral bioavailability (Faderl *et al.* 2005).

Clinically, clofarabine is used as a second line treatment to treat relapsed acute lymphoblastic leukaemia in children, who have received at least two other forms of treatment. However, it is being put through various clinical trials to treat adult lymphoblastic haematological malignancies and myelodysplastic syndromes (Faderl *et al.* 2005).

Like other drugs of this class, clofarabine requires transport across the membrane via ENTs and CNTs, but, over long periods of time, it can diffuse across the plasma membrane (King *et al.* 2006). Intracellular phosphorylation is again required by purine kinases, particularly dCK to the mono-, di- and triphosphate forms of the drug, with the latter being the most important anti-cancer form of the drug. Lotfi *et al.* (1999) demonstrated that the efficiency in terms of V_{max}/K_m, of clofarabine phosphorylation by dCK was 2- and 8-fold higher compared to cladribine and its natural substrate, 2'-deoxycytidine, respectively. Similarly, Parker *et al.* (1999) demonstrated increased efficiency of clofarabine phosphorylation by dCK compared to cladribine, fludarabine and 2'-deoxycytidine. Clofarabine can self-potentiate itself by inhibition of ribonucleotide reductase causing a decrease in intracellular 2'-deoxycytidine levels, which is a feedback inhibitor of dCK.

Clofarabine has mechanisms of action similar to cladribine and fludarabine and is shown in Figure 1.6. Firstly inhibition of ribonucleotide reductase causes dNTP pool depletion. This then increases inhibition of DNA polymerase α and ε and encourages clofarabine triphosphate insertion into terminal DNA strands resulting in impairment of DNA elongation and repair and DNA strand breakage. These mechanisms induce apoptosis, but clofarabine can also act directly on the mitochondria resulting in altered transmembrane potential and release of several apoptotic molecules into the cytoplasm including cytochrome C, apoptotic-inducing factor (AIF), caspase 9 and apoptosis protease-activating factor 1 (APAF1) (Xie and Plunkett 1996; Genini *et al.* 2000a; Bonate *et al.* 2006).

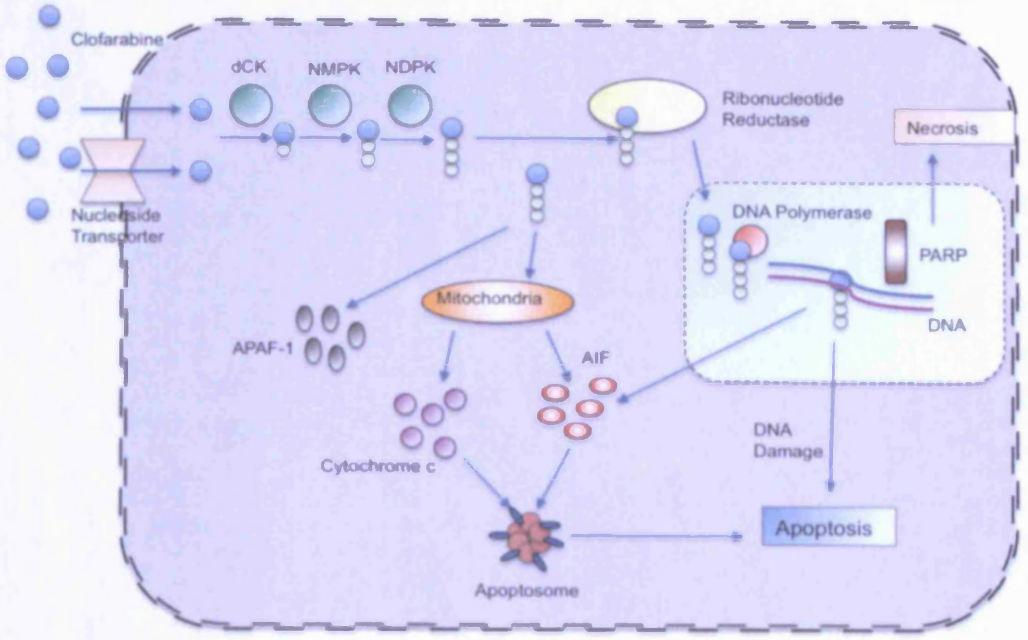


Figure 1.6 Mechanisms of Action of Clofarabine.

Clofarabine is transported intracellularly by NTs or passive diffusion and is then successively phosphorylated by dCK and then nucleoside mono- and diphosphate kinases (NMPK and NDPK, respectively) to clofarabine triphosphate (ClofTP). ClofTP inhibits ribonucleotide reductase, decreasing cellular dNTP pools and causing ClofTP to outcompete dATP. ClofTP inhibits DNA polymerase and is incorporated into DNA strands inhibiting DNA synthesis and repair and causing DNA damage. This leads to activation of PARP which leads to depletion of NAD^+ causing necrosis and also leads to initiation of apoptosis. ClofTP can also act directly on the mitochondria causing release of cytochrome c, apoptosis inducing factor (AIF) and apoptotic protease activating factor 1 (APAF 1) leading to apoptosis.

1.4.6 Specific Mechanisms of Action

1.4.6.1 Inhibition of Ribonucleotide Reductase

Ribonucleotide reductase is an essential enzyme that produces deoxyribonucleotides diphosphates directly via reduction of the hydroxyl on the ribose moiety of ribonucleotide diphosphates (Cerqueira *et al.* 2005), and is a rate-limiting enzyme in DNA synthesis. Ribonucleotide

reductase is an essential enzyme for cell viability as it was found that cells were unable to produce deoxyribonucleotides directly from cell metabolism, but have to be synthesised from ribonucleotides directly via reduction by ribonucleotide reductase (Cerqueira *et al.* 2005). Inhibition of ribonucleotide reductase is one of the principle mechanisms of action of NAs.

There are two subunits of ribonucleotide reductase, termed RRM1 and RRM2 (Cerqueira *et al.* 2005). The RRM1 subunit is an 85 kDa protein that contains the active site for substrate binding, as well as the allosteric regulatory site. The RRM2 subunit is 45 kDa and houses the diferric iron centre and tyrosyl free radical that are involved in substrate reduction reactions (Uhlin *et al.* 1994; Jordan and Reichard 1998). The RRM1 unit is usually termed the regulatory subunit, whereas RRM2 is considered the catalytic subunit. Transcriptional regulation of ribonucleotide reductase is controlled through cell cycle-specific induction. The RRM1 subunit is constitutionally expressed and has a half–life of approximately 18-24 hours. RRM2 transcription, however, is S-phase specific and has a maximal half-life of 4 hours and is degraded rapidly during mitosis (Bjorklund *et al.* 1990; Cerqueira *et al.* 2005). Enzyme activity is controlled through allosteric regulation. Binding of ATP at the active site stimulates enzyme activity, whereas dATP binding is inhibitory to function. Binding of allosteric effecters including ATP, dATP, thymidine triphosphate and deoxyguanosine triphosphate control the overall function of the enzyme stimulating reduction of cytidine, uracil, guanosine and adenosine diphosphate, respectively (Jordan and Reichard 1998). Inhibition of ribonucleotide reductase therefore causes depletion of the cellular dNTP pools, which inhibits DNA replication and repair, and encourages incorporation of NAs into DNA strands.

Tanaka *et al.* (2000) first described a p53-dependent protein that was expressed in p53-wild type but not in p53-mutant colorectal

adenocarcinoma (SW480) cell lines. This protein shared 80% sequence homology with RRM2 throughout its structure, except for the N-terminal region containing the KEN box that enables RRM2 to be recognised and induced by Cdh-1-anaphase promoting complex and as a result, p53R2 can be induced throughout the cell cycle (Chabes *et al.* 2004). p53R2 expression is induced by p53 activation in response to DNA damage such as chemotherapeutic agents or UV radiation and functions to help repair DNA by the supply of dNTPs (Tanaka *et al.* 2000). Upon activation, p53R2 forms a complex with the RRM1 subunit by binding to the same site and functioning in place of RRM2, although with less affinity and resulting in lowered overall enzymatic activity (Guittet *et al.* 2001; Shao *et al.* 2004). After activation, the p53-p53R2 complex disassociates and p53R2 translocates to the nucleus where it binds with the RRM1 subunit to provide dNTPs (Yamaguchi *et al.* 2001; Xue *et al.* 2003). p53R2 also functions to protect cells against oxidative stress, as it has been found to have catalase activity (Xue *et al.* 2006).

1.4.6.2 Inhibition of DNA Polymerase and DNA Strand Breakage

DNA polymerase is a key enzyme in DNA synthesis and repair. It is responsible for the polymerization of deoxyribonucleotides during DNA strand synthesis. NAs including cladribine, fludarabine and clofarabine compete with dATP for the binding site in DNA polymerase. Clofarabine is an inhibitor of α and ε subtypes but not β or γ (Bonate *et al.* 2006), which then incorporates these drugs as the monophosphate into the DNA strand. DNA elongation and repair is then inhibited when it comes to a site of a NA rather than a normal nucleotide, which cause chain termination and DNA strand breakage.

1.4.6.3 Inhibition of SAH Hydrolase

Inhibition of ADA by deoxycoformycin causes an accumulation of 2'-deoxyadenosine which in turn inhibits the enzyme SAH hydrolase. This leads to an increase in intracellular SAH concentration (Fidias *et al.*

1996). Accumulation of SAH inhibits methyltransferases, which results in an inhibition of essential methylation reactions involved in the epigenetic regulation of DNA, RNA and proteins (Hermes *et al.* 2008) leading to p53-dependant apoptosis (Jackson-Grusby *et al.* 2001; Karpf *et al.* 2001). Interestingly, a study by Wyczechowska and Fabianowska-Majewska (2003) found that fludarabine and cladribine were also able to inhibit SAH hydrolase resulting in hypomethylation and a reduction in DNA synthesis in a human myelogenous leukaemia cell line (K562). Similarly, Hermes *et al.* (2004) identified that adenosine-mediated inhibition of SAH hydrolase caused upregulation of various p53-target and apoptosis-inducing genes.

1.4.6.4 Activation of PARP

PARP are a family of enzymes involved in DNA repair and apoptosis. PARP enzymes function to signal DNA damage to the cell by binding to the ssDNA or dsDNA breakage via zinc fingers. Then, using nicotinamide adenine dinucleotide (NAD^+) as a substrate, PARP attaches multiple ADP-riboses to DNA repair proteins, DNA and histones, ultimately leading to unwinding of the damaged DNA and its repair via the base excision repair pathway (Ratnam and Low 2007). Indeed, over activation of PARP leads to cellular necrosis via depletion of NAD^+ and thus energy stores of the cell, but it has also been demonstrated to induce cell death by apoptosis via the release of AIF from the mitochondria (Yu *et al.* 2002). Both fludarabine and cladribine have been shown to induce PARP activation due to their mechanisms of action on initiation of DNA damage that ultimately leads to this NAD^+ depletion and cell death and is thus suggested as a cytotoxic mechanism in non-dividing cells. However, Pettitt *et al.* (2000) found in patients with leukaemia that whilst the activation of PARP was able to induce cell death in occasional cases, activation of PARP instead contributed to the acceleration of cell membrane disruption in the later stages of apoptosis in response to fludarabine. They also noted that

PARP-associated cell death was second only to dysfunctional p53-dependent cell death as this was the most rapidly observed cell death pathway.

1.4.6.5 p53-Dependent and Independent Induced Apoptosis

The tumour suppressor, p53, is the quintessential tumour suppressor and is one of the most widely studied molecules involved in cancer, cell cycle, apoptosis and ageing. Coded for by the TP53 gene, the protein is often termed the 'guardian of the genome' and is known for its ability to suppress cancer cell genesis. DNA damage is able to induce activation of p53, which can either permanently halt cell cycle progression (cellular senescence) or induce apoptosis of the damaged cell before it turns cancerous. The importance of p53 in halting the neoplastic transformation of damaged cells is demonstrated by the fact that 50% of all human cancers have a loss of or mutated forms of p53 that render it inactive (Efeyan and Serrano 2007). Literature suggests that loss of p53 function either by mutation or total loss of the protein is associated with poorer prognosis, due to these cells displaying increased resistance to DNA damaging agents (Sturm *et al.* 2003).

Pettitt *et al.* (1999) using a p53 knockout splenic cell model, found cladribine and fludarabine were able to induce apoptosis in both a p53-dependent and -independent fashion, albeit, p53 knockout cells were more resistant to the nucleosides. Galmarini *et al.* (2003a) showed that in colorectal carcinoma cells (HCT116) that express wild type p53 protein, cladribine was able to induce apoptosis by p53 activation, cell cycle arrest and PARP and Bax overexpression. Similarly, they found that p53 negative cells were more resistant to cladribine's toxicity.

1.5 Breast Cancer

1.5.1 Breast Anatomy

The human breast describes the mammary glands that function physiologically and developmentally as a source of nutrition for the young, being only functional in females. Each breast itself is positioned anterior to the chest wall and lies within the superficial fascia of the pectoral muscles and subcutaneous fat of the skin. A central areola and nipple forms the site of lactation. Histologically, the breast is made up predominately of connective, glandular epithelial, and adipose tissues which make up the four essential structures; lobules, ducts, fatty tissue and supportive/connective tissue (Mostafa *et al.* 2006). Each breast consists of between 15-25 lobes that are arranged in a radial fashion around the central nipple region, with each lobe separated by fatty and connective tissue and suspensory ligaments. Within each lobe, numerous lobules containing glandular alveoli produce milk during lactation. The milk is secreted into the lactiferous ducts that protrude through the lobes, whereby they pass through the lactiferous sinus that open to the nipple (Marieb *et al.* 2004).

1.5.2 Breast Cancer Epidemiology

In the UK there are over 44,000 new cases of breast cancer diagnosed annually. Worldwide, this number rises to over 1 million new cases and the majority of these are in more affluent countries. Despite the steady decline in breast cancer mortality rates over the last 10 years, some 12,000 breast cancer deaths occur annually in the UK and 1,400 of these deaths occur in women under 50. This earlier onset breast cancer tends to be associated with an ER-negative and significantly more aggressive phenotype with a poorer prognosis and is a major clinical problem where new treatment options are needed.

1.5.3 Breast Cancer Sub-Types, Classifications and Staging

Breast cancer refers to a number of different neoplasms of the breast tissue and there are now numerous ways to identify and type breast cancers, based upon histology, molecular biology or genetic markers. Nearly all cancers of the breast are carcinomas or adenocarcinomas, i.e. they arise from the epithelial or glandular epithelial tissue, respectively. Connective tissue cancers (mostly angiosarcomas) are extremely rare in the breast, and most cases occur in patients who have previously had a carcinoma of the breast, and/or radiation therapy (Cozen *et al.* 1999).

Classifying breast cancers, and providing information on their histology and molecular status provides important prognostic information and is used to determine the treatment options. There are varying ways to classify and group breast cancers, usually relying on morphological and histopathological assessment, hormone and molecular marker status (Elston *et al.* 1999). A relatively recent method looks at the molecular sub-type of breast cancer by identifying patterns of genes and associating them with being basal or luminal and various classes in between.

1.5.3.1 Pathological Classification

Pathological classification of breast cancers uses traditional morphological aspects of pathology including tumour size, differentiation (including histological type and grade), lymph node involvement and vascular invasion. There are different systems for this, usually employing the TNM system from the American Joint Cancer Committee on Cancer (tumour size; T, number of nodes involved and location; N, and presence or absence of metastasis beyond the regional lymph nodes; M) or the Nottingham Prognostic Index (NPI) which looks at tumour size, histological status and lymph node involvement and is widely used in the UK and Europe (Yu *et al.* 2004). Although extremely

valuable for determining clinical outcomes, there are limitations to these systems as grading and analysis of tumours is subject to inherent personal bias and variability.

1.5.3.2 Lymph Node Involvement and Vascular Invasion

Pathological classifications are used to determine the extent of the tumour within the body. Nodal involvement is much more widely used and of more prognostic value than vascular invasion. The NPI simply uses the terms 'unspecified' or 'vascular invasion' to classify the presence of tumour emboli in the circulatory system as its value is still under debate although it is becoming a more important prognostic factor as more is understood (Elston *et al.* 1999). Lymph node involvement with metastatic tumours is associated with prognosis, and is used in the TNM and NPI systems. Those patients showing no nodal involvement have a 75% chance of surviving past 10 years, compared to only a 25-30% chance in those showing metastases (Elston *et al.* 1999).

1.5.3.3 Tumour Size

Generally, patients with a smaller tumour size upon presentation are suggested to have a better prognosis than those with larger tumours, regardless of other factors. Tumours termed minimal invasive carcinoma, are tumours that are less than 9 or 10 mm in diameter and are associated with an excellent prognosis (Elston *et al.* 1999).

1.5.3.4 Differentiation

Tumours are now classified based on their differentiation, that is how far the tumour cells are differentiated from their normal, non-cancerous tissues from which they derived. Tumours are not just broadly split into *in-situ* or invasive but looks at histological type and histological grade. Histological type refers to the architectural pattern of the tumour and its origins and can be classified into the categories below.

1.5.3.4.1 Carcinoma *In-Situ*

The term carcinoma *in-situ* is a frequently used term meaning a benign tumour and is preceded by either ductal or lobular, referring to the origin of the cells. The term refers to increased hyperplasia, i.e. an increase in cell number, where the cells are within the confines of the duct or lobule accordingly, and is an indicator of likely progression to a malignant cancer (Hammer *et al.* 2008).

1.5.3.4.2 Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is a relatively rare form of breast cancer, and accounts for between 1-6% of all new breast cancer cases annually (Kleer *et al.* 2000). IBC is amongst the most aggressive type of breast cancer and is distinctly different from other breast cancers as the clinical symptoms do not mach those of other breast tumours. There is usually no palpable lump or tumour mass, instead cancer is spread throughout the breast and, in nearly all cases, there is lymph node metastasis, with about a third showing distant metastasis (van Golen *et al.* 2000). The appearance of the breast differs from that of classical breast carcinoma, exhibiting signs of inflammation including redness, swelling, firmness and soreness. These symptoms are rapidly progressing, usually within 2 months of initial onset. Primary IBC refers to a patient with an underlying carcinoma of the breast, but this usually remains undetectable at the time of diagnosis. Secondary IBC is the result of reoccurrence of inflammation of a non-inflammatory breast carcinoma (Kleer *et al.* 2000).

1.5.3.4.3 Invasive Ductal or Lobular Carcinoma of the Breast

Most incidences of breast cancer are invasive ductal or lobular carcinoma (IDC and ILC, respectively). Invasive mixed ductal-lobular carcinoma (IDLC) are tumours that express mixed populations of ductal or lobular cells and the origin of the tumour is undecipherable. IDC is the most common form of cancer, accounting for between 70-80% of

breast cancers (Berg and Hutter 1995). ILC, however, accounts for only 15% of breast cancers. Clinically, ILC is more likely to be hormone-receptor positive and is generally associated with better clinical outcomes and increased survival rates compared with IDC (Li *et al.* 2003).

There are other forms of breast cancer that do not fall into the above categories, but these tend to be very rare and account for <1% of breast cancers each. These may include medullary, tubular, cribiform or mucinous breast cancers.

1.5.3.5 Histological Grading

Tumour grading is based upon the Scarf-Bloom-Richardson system, which takes into account nuclear grade, tubule formation and mitotic index and assigns a score based upon these. The Nottingham variation of this is now becoming the most widely used system in the UK, Europe and America. This method gives a score of 1-3 for each category with 1 being the best and 3 being the worst. The scores are tallied and used to determine the 'grade' or degree of differentiation of the tumour. A high grade, poorly differentiated tumour (the cancer cells are very different to the normal cells from which they derived; Grade III) is associated with a more aggressive tumour and poorer clinical prognosis. Tumours that are low grade and are well differentiated (the cancer cells are very similar to the normal cells they are derived from; Grade I) have a better overall prognosis. Indeed it is suggested that those with grade I tumours, have an 85% 10 year survival rate, compared to a 45% 10 year survival rate for those with grade III tumours (Elston *et al.* 1999).

1.5.3.6 Hormone Status

The hormone status of breast cancers is widely used to determine prognostic information, but also used to dictate treatment regimes. Breast cancer, as well as other cancer types such as prostate or ovarian cancer, can be responsive to the actions of various hormones,

and deprivation of these hormones can reduce the growth of these tumours. In breast cancer, hormone status usually refers to oestrogen-receptor alpha (ERα) status and progesterone-receptor (PR) status, being positive or negative for detectable amounts of the protein. ERβ status is not usually used to classify breast tumours (Rochefort *et al.* 2003).

Upon presentation, approximately 60-70% of breast tumours are ER-positive and the presence or absence of detectable ER is an important prognostic indicator (Brinkman and El-Ashry 2009). Those who initially present as ER-positive are likely to encounter longer disease-free survival and an overall better prognosis than those with an ER-negative phenotype (Rochefort *et al.* 2003). ER status determines treatment options, with those being ER-positive, likely to benefit from endocrine therapies such as tamoxifen or faslodex. However 25-30% of ER-positive tumours do not respond to tamoxifen initially, and most will go on to develop resistance in the future (Brinkman and El-Ashry 2009). Those with the ER-negative phenotype will generally not respond to endocrine therapy and its use is not advocated.

1.5.3.7 Oestrogen and the ER

Oestrogens are steroid hormones primarily found in females that serve to regulate the growth and physiological processes of the reproductive system. They also have influence over pathological process, particularly hormone-dependent cancers such as breast, ovarian, prostate and endometrial (Chen *et al.* 2008). The most predominant of this class of hormone is 17β-oestradiol (oestrogen), which is regulated by 17β-hydroxysteroid dehydrogenase, an enzyme that converts oestrogen to its less active metabolite, oestrone (Bai and Gust 2009).

The physiological activity of oestrogen is modulated by binding to the oestrogen receptors α and β (Katzenellenbogen *et* al. 2000). In breast cancer, ERα is suggested to be the predominant receptor in activation

of oestrogen-responsive genes (Nicholson and Johnston 2005). The ERs belong to the nuclear receptor superfamily, a class of ligand-regulated transcriptional factors (Matthews and Gustafsson 2003). Both isoforms of ER share significant homology, both consisting of domain regions A-F, where region C is associated with DNA binding and receptor dimerisation. Upon binding of the ligand to the ER, the receptor undergoes significant conformational changes and the disassociation of various heat shock proteins including hsp90 and hsp70 (Klinge 2001) to allow it to dimerise with other ligand-bound ERs (Yang *et al.* 2008).

The oestrogen-ER complex can then influence transcription by two mechanisms: binding of the oestrogen-ER complex to a specific nucleotide sequence found in the promoter regions of oestrogen-responsive genes called the oestrogen response element, aided by zinc fingers on the oestrogen-ER complex that sit into the major grove of the DNA helix (Klinge 2001) or by binding of the oestrogen-ER complex to another DNA-bound transcription factor such as AP-1 that will stabilize the binding of that transcription factor to the DNA. Both methods result in the recruitment of coactivators and activation of the transcriptional machinery activating genes associated with cell or tumour growth (Klinge *et al.* 2004).

1.5.3.8 Molecular Markers

Molecular markers including over- or underexpression of various genes and their respective proteins offer a new way of classifying breast cancers and are able, in combination with pathological and hormonal status, to determine prognostic information and suggest treatment options (Esteva and Hortobagyi 2004). Some of these markers include the epidermal growth factor receptor 2 (EGFR2; Her2), Ki-67, *c-myc,* p53 and E-cadherin (Elston *et al.* 1999). Of these, Her2 is arguably the most important in determining treatment options and prognosis, although the others still have value. Ki-67 for example, is a nuclear antigen that is expressed during proliferative stages of the cell cycle,

but not under resting phase (G0). Those patients who exhibit high indices of Ki-67 compared to low expressing tumours demonstrated a higher disease reoccurrence and shorter survival times (Veronese *et al.* 1993).

1.5.3.9 Her2 Status

The most widely used molecular marker in breast cancer classification is Her2 status. This and other members of this type 1 superfamily, including EGFR, Her3 and Her4, belong to the tyrosine kinase receptors. In normal cells, expression of Her2 found on chromosome 17 encodes a 185-kDa transmembrane protein, with tyrosine kinase activity. Via heterodimerisation with other members of the EGFR family, Her2 mediates signal transduction pathways involved in cell proliferation and differentiation (Guan *et al.* 2005). Overexpression of the Her2 gene causes an increase in the number of receptors on the cell, which causes excessive receptor activation and a subsequent increase in cell proliferation, division and eventually tumour formation. Her2 overexpressing breast cancer tumours are associated with a more aggressive phenotype, higher tumour grading and ultimately, shorter disease-free and overall survival rates (Hicks and Kulkarni 2008). Approximately 25% of breast tumours present with this over-expression of Her2 (Hicks and Kulkarni 2008). However, those who are Her2 overexpressing are likely to respond to the monoclonal antibody therapy, trastuzumab (herceptin) with good results. Trastuzumab has been shown to downregulate expression of Her2, most likely by increasing receptor endocytosis and degradation (Molina *et al.* 2001).

1.5.3.10 Basal and Luminal Classification

With the advent of increasingly better biotechnology, specifically microarray technology, a relatively new system of breast cancer classification has arisen. This method looks at the expression of a cohort of genes and classifies breast cancers into a certain group based

upon over or underexpression of gene clusters. The pioneers of classifying breast cancers in this way were Perou *et al.* (2000) who looked at the expression of 500 genes in normal and breast cancer tissues and were able to show clear correlations of gene clusters in individual samples. Subsequent work has now identified five sub-types, luminal A and B, basal, Her2 overexpressing and normal-like (Adélaïde *et al.* 2007).

Luminal A and B are ER-positive and share features with luminal cells of the mammary ducts. The normal-like sub-type demonstrates increased expression of basal-genes, but reduced expression of luminal-associated genes. The basal subtype shares characteristics with normal breast basal epithelial cells, but are generally devoid of ER and PR and are not Her2 overexpressing and are termed 'triple-negative'. These tumours originate from the basal layer of the mammary duct epithelium (Cleator *et al.* 2007). Basal-like tumours express high molecular weight cytokeratins associated with basal cells, including CK, CK5/6, CK14 and CK17 (Reis-Filho and Tutt 2008). A small number of basal-like subtype cells do express some of these receptors, however, suggesting there is not a complete correlation with basal and triple negative breast cancers (Cleator *et al.* 2007). Luminal A breast cancer is associated with a good overall prognosis. Luminal B, basal and Her2 overexpressing breast cancers, however, are associated with a much poorer prognosis, with Her2 overexpressing being the only group with a specific targeted therapy in the form of trastuzumab (Adélaïde *et al.* 2007).

1.5.3.11 ER-Negative and Triple-Negative Breast Cancer

ER-negative breast cancers, although they are less frequent then their ER-positive counterparts, are associated with a more aggressive phenotype, although the reasons for this are still largely unknown (Rochefort *et al.* 2003). ER-negative cancers can arise from ER-positive premalignant lesions that undergo genetic or epigenetic alterations in

the ER gene regions, or have an increased degradation of ER proteins. ER-negative cancers are suggested to arise directly from a hormone-independent pathway (Rochefort *et al.* 2003). Those patients defined as ER-negative (which clinically refers to ERα status) encompasses those with a poorer prognosis, and limited treatment options available compared to those with ER-positive cancer (Skliris *et al.* 2008). However, those with ER-negative, but Her2 overexpressing tumours have targeted therapies available with trastuzumab (Doane *et al.* 2006).

Triple-negative breast cancers (TNBCs) account for approximately 15-20% of all breast cancers (Cleator *et al.* 2007). This group is similar to the basal subtype described by genetic fingerprinting, but not all basal termed tumours are triple negative. Triple negative and basal-like tumours share certain clinical characteristics that differentiate them from other tumour types including increased incidence in African-American women, often affecting younger women less than 50 years old, and being considerably more aggressive than other subtypes. Clinically this relates to reduced 5 and 10 year survival rates compared to non-basal or ER-positive/Her2 overexpressing tumours (Reis-Filho and Tutt 2008). Patients with TNBC are also more likely to see disease reoccurrence both locally and with distant metastasis than those with ER-positive or Her2 overexpressing tumours (Dent *et al.* 2007). These clinical outcomes are expected as most triple-negative and basal-like tumours are associated with histological grade 3, known to be correlated with the worst clinical outcome (Elston *et al.* 1999; Reis-Filho and Tutt 2008).

1.6 Current Treatments in Breast Cancer

Treatment options for breast cancer are varied and determination of the appropriate treatment for the individual patient is decided by the patient's condition i.e. extent of the disease, age, general health and menopausal status; as well as the subtype of breast cancer present, particularly steroid hormone receptor status and Her2 status. Treatment nearly always begins or involves at some stage a mastectomy or

surgery to remove the tumour and help define the cancer stage (Hammer *et al.* 2008). This is then followed by chemotherapy and endocrine therapy to reduce the chance of metastasis, and radiotherapy to reduce the risk of recurrence (Ismaili *et al.* 2009). Neoadjuvant treatment is sometimes given to reduce the size of the tumour prior to surgical intervention in order to increase the success of the surgery. However, whether or not neoadjuvant therapy leads to improved disease-free survival and better prognosis overall is still under debate, and neoadjuvant therapy is only given to select patients, particularly those with large tumours (Smith 2008).

1.6.1 ER-positive Breast Cancer: Endocrine Therapy

Endocrine therapy is based upon the reasoning that most breast cancers rely on the growth-promoting effects of oestrogen to proliferate. Endocrine therapy therefore lends itself in two ways; ablative therapy to remove the source of the oestrogen and additive therapy to act upon the cancer itself to prevent the proliferative action of oestrogen, primarily through antagonising the oestrogen receptor. Approximately 60-70% of breast tumours are ER-positive and its presence strongly supports the use of endocrine therapy (Brinkman and El-Ashry 2009).

Ablative therapies can refer to surgery or chemotherapy. In surgery, the glands that produce oestrogen are removed and thus the source of endogenous oestrogen is removed. In premenopausal women these are the ovaries, in postmenopausal women these are the adrenal glands. In postmenopausal women, oestrogen can still be produced by aromatisation of circulating hormones, particularly testosterone and androstendione to oestrogen and oestrone. This reaction is catalysed by the enzyme aromatase, which controls the rate-limiting step in oestrogen synthesis (Macedo *et al.* 2009). Further to this, two-thirds of breast cancers express the aromatase enzyme and are likely to synthesise their own oestrogen (Lipton *et al.* 1987). The development of aromatase inhibitors has progressed rapidly, and the current, third

generation drugs include anastrozole (Arimidex), letrozole (Femara) and exemestane (Aromasin). These drugs exhibit high selectivity and potency for aromatase and, such is their success, they have now replaced tamoxifen as a first-line therapy against ER-positive breast cancer (Macedo *et al.* 2009; NICE Guidelines 2006).

Additive therapies are largely based upon anti-oestrogens, being well described for the treatment of ER-positive, oestrogen-dependent breast cancers. These act to stop the proliferative effects of oestrogen at the cancer cell itself. The selective oestrogen receptor modulator (SERM), tamoxifen, had been the gold standard in treating ER-positive breast cancer for over 30 years (Schwartzberg *et al.* 2009). Indeed, endocrine therapy offers the advantages of conventional cytotoxic chemotherapy but without many of the unwanted side effects (Nicholson and Johnston 2005). Tamoxifen therapy has its own drawbacks however, including endometrial hypertrophy, endometrial cancer and an increased risk of venous thrombi, all due to the partial agonist activity of tamoxifen for the ER (Patel *et al.* 2007). Although the benefits of tamoxifen far outweigh the risks for most people, the major limitation in its use is the development of acquired resistance to the drug (Goss and Strasser 2002), which results in relapse of the cancer and ultimately, patient death.

The need for oestrogen receptor antagonists without agonist activity was evident, however, and led to the development of a novel oestrogen analogue, fulvestrant (faslodex). Faslodex is devoid of agonist activity and binds to the ER with similar affinity to oestrogen itself (Nicholson and Johnston 2005). As well as being able to antagonise the ER and thus inhibit oestrogen signalling, faslodex is able to impair receptor dimerisation and thus reduce translocation of ER to the nucleus. As the faslodex-ER complex is unstable, this hastens the degradation of the ER protein, resulting in down-regulation of available ER (Osborne *et al.* 2004).

All patients on tamoxifen, whilst responding well initially will go on to develop a degree of resistance, characterised by continued growth of the tumour in the presence of the drug. Some tumours will, however, respond to second or third line endocrine therapy, but not all and as such, resistance to endocrine treatments is a major clinical problem (Larsen *et al.* 1997). One of the other major drawbacks in endocrine therapy is *de novo* resistance to endocrine therapy from the start, mainly due to the absence of functional ER expression (Nicholson and Johnston 2005).

1.6.2 Her2 Overexpressing Breast Cancer: Trastuzumab Therapy

Her2 overexpressing tumours account for approximately 25% of reported breast cancers, and although Her2 overexpression suggests a more aggressive cancer, specific targeted therapies have been developed against this target, namely trastuzumab. Trastuzumab is a humanized monoclonal antibody directed against the Her2 protein. Trastuzumab was given FDA approval for the treatment of Her2 overexpressing metastatic breast cancer in 1998, and was active as both a single agent and in combination with standard chemotherapy (Nahta and Esteva 2006). However, most tumours will go on to acquire resistance within a year. Treatment with adjuvant chemotherapy of paclitaxel or docetaxel can reduce acquired resistance and lead to longer disease-free survival (Nahta and Esteva 2006).

The Her2 status has been suggested to influence response to various chemotherapy treatments. Indeed, Her2 overexpressing tumours have been demonstrated to show increased sensitivity to doxorubicin and anthracycline chemotherapy, as well as other chemotherapeutics including cyclophosphamide and 5-fluorouracil (Muss *et al.* 1994; Paik *et al.* 1998; Thor *et al.* 1998). However, response to the taxanes including paclitaxel does not appear to be correlated with Her2 overexpression (Van Poznak *et al.* 2002).

The interaction between ER and expression of Her2 is still under review. However, Her2 and the ER co-activator AIB1 are often elevated together, and there are data to show crosstalk between the ER and Her2 pathways (Shou *et al.* 2004). A study by Lipton *et al.* (2002) demonstrated that patients who are ER-positive and Her2 overexpressors, were less likely to respond to anti-hormone therapy than their Her2 normal-expressed counterparts. This also correlates to shorter survival times with similar results found by Arpino *et al.* (2004).

1.6.3 Treatment of ER-negative and TNBC

For those who have either ER-negative or Her2 normal-expressed tumours, treatment is generally based upon the other target i.e. in ER-negative but Her2 overexpressing, trastuzumab, and SERMs/aromatase inhibitors with ER-positive but Her2 normal expressed tumours. Depending upon the stage, spread and histological grade/classification of the disease, these treatments may be backed up with adjuvant cytotoxic chemotherapy also. However, for those who lack expression of ER and are Her2 normal, standard cytotoxic chemotherapy is the only option after surgery an/or radiation. Current chemotherapy treatment of TNBC patients relies on combinations of cytotoxic agents, although there is no standard regime for this (Cleator *et al.* 2007).

Triple-negative and basal like tumours are highly sensitive to anthracycline and taxane chemotherapy, especially when combined. In fact, triple-negative and basal-like tumours often show increased sensitivity to cytotoxic chemotherapy compared to luminal A, or hormone-responsive tumours. However, despite this increased initial sensitivity, disease free and overall survival remain poorer (Anders and Carey 2008).

Due to the linkage of TNBC and dysfunctional DNA repair mechanisms, the use of DNA damaging agents could be indicated as a treatment

option. Recent data has suggested the use of platinum-based treatments, which are DNA damaging agents, such as cisplatin encouragingly show sensitivity of these tumours to platinum-based agents. However, to date no optimal regimes have been established for their use and they remain in trial stages (Gluz *et al.* 2009).

For those who are Her2 normal-expressed, treatments consist generally of standard regimes of multiple cytotoxic agents including cyclophosphamide, 5-fluorouracil, doxorubicin, methotrexate or epirubicin (EBCTCG, 2008). These drugs belong to the DNA replication/inhibitor class, and are folic acid inhibitors (methotrexate), alkylating agents (cyclophosphamide), topoisomerase inhibitors (doxorubicin, epirubicin) or pyrimidine thymidylate synthase inhibitors (5-fluorouracil). Although these drugs are effective against cancer, the biggest limitation is lack of specificity and the side effects of the drugs as they also act on other tissues, specifically fast-dividing cells such as those found in the mucosa and epithelial linings. Fortunately, ER-negative breast cancers are often associated with being Her2 overexpressed and may benefit from trastuzumab therapy (Doane *et al.* 2006). Although anthracyclines and taxanes are effective against ER-negative and TNBCs, their main drawback is the development of severe side effects, in particular heart failure and the development of leukaemia (Shapiro and Recht 2001; Nowak *et al.* 2004).

1.6.4 Why NAs as Treatments for Breast Cancer?

1.6.4.1 Selectivity

Modern cancer research is directed at targeted therapies to specific cancer cell traits. A drawback of these methods is that finding a target is more challenging i.e. does every type of cancer express a particular protein. What makes PNAs different from standard chemotherapeutics is their degree of selectivity for malignant cells over normal tissue.

As NAs are prodrugs and require phosphorylation by dCK, malignant cells expressing high dCK levels compared to non-malignant tissues are likely to be more susceptible to the action of NAs (Eriksson *et al.* 1994; Spasokoukotskaja *et al.* 1995; van der Wilt *et al.* 2003).

Similarly, malignant cells of solid tumours have higher expression of NTs compared to normal, surrounding tissue (Mackey *et al.* 1998; Zhang *et al.* 2007) making these cells more susceptible to the action of NAs (Pennycooke *et al.* 2001).

There are contrasting studies on the expression of the metabolising enzyme cN-II between malignant and normal tissue (Rosi *et al.* 1998a and 1998b; Vannoni *et al.* 2004). It is difficult to interpret whether the expression of cN-II between malignant and non-malignant tissues can influence selectivity of NAs (Hunsucker *et al.* 2005)

1.6.4.2 Drug Tolerance

PNAs have minimal non-haematological associated toxicities that makes them excellent agents when considering the effects on a patient's quality of life. The minimal toxicities also make them attractive candidates to be considered in multi-drug regimes for patients (Rummel *et al.* 2002).

Guchelaar *et al.* (1994) suggested that cladribine is a well-tolerated drug, and that side effects including nausea, vomiting, alopecia, stomatitis and organ toxicity are mild to absent. Robak *et al.* (2006) states that cladribine has distinguishable toxicities compared to other cytotoxic agents that commonly include nausea, vomiting, hair loss and abnormal liver or kidney function and these are rarely reported. Similarly, at standard doses, neurotoxicity is also rare. Studies in patients with multiple sclerosis, for which cladribine is being investigated, suggests that cladribine at standard chemotherapeutic doses is remarkably well tolerated, with even limited myelosuppression observed (Selby *et al.* 1998). These data are shown clinically as

demonstrated by a case report of a woman with hairy cell leukaemia, in which the authors state that cladribine is drug of choice for hairy cell leukaemia because of the ease of administration, it is well tolerated and has minimal side effects (Seshadri *et al.* 2000).

Jeha *et al.* (2004) found that clofarabine was well tolerated in children with relapsed acute lymphatic leukaemia, and, importantly for its implications in breast cancer, is well tolerated even in the elderly (Pui *et al.* 2005). A study by Cooper *et al.* (2004) identified that clofarabine was again well tolerated and the dose-limiting toxicity was myelosuppression. However, the number of patients in this group was small (n=13) and the solid tumours were not defined, although they were stated to be metastatic.

1.7 Conclusions

Although treatments for breast cancer have come a long way as suggested by the reduced incidence and mortality of breast cancer, there is still an obvious need to develop further treatments. Whilst treatments for those women who are ER-positive, or who overexpress Her2 with aromatase inhibitors or trastuzumab, respectively, are well established and achieve good response rates, there is a subset of women who benefit little from these methods of therapy and instead rely on standard cytotoxic chemotherapy (Howell *et al.* 2005). Those termed triple-negative, although making up a small number of breast cancer patients, have limited treatment choice and very poor prognosis (Doane *et al.* 2006).

Targeted therapies are the mainstay of research in the modern era, but the effectiveness of cytotoxic agents cannot be ignored. Their limitations however, including toxicity towards other tissues and side-effects, are a major drawback and can drastically reduce quality of life for patients on these therapies. The PNAs are a unique sub-set of cytotoxic agents in that they can offer some selectivity over standard

cytotoxics, which also contributes to them being well tolerated and thus having reduced side effects. Another major advantage of using PNAs is that they are already in the clinic and their usage and safety profiles are well documented, with clinicians having wide experience in their use. This could potentially lead to the treatments being available to patients much sooner, and having the advantage of clinicians knowing the most effective regimes for these drugs.

1.8 Thesis Aims

The principle aims of this thesis were to investigate the activity and mechanisms of action of adenosine, 2'-deoxyadenosine and their clinically used analogues in breast cancer cell lines *in vitro*.

The main objectives for this thesis include:

- To investigate the expression and role of adenosine receptors in breast cancer cell proliferation.
- To determine what effects manipulating endogenous and exogenous adenosine and 2'-deoxyadenosine would have on the viability of breast cancer cells
- To investigate the pharmacological activity of clinically used purine analogues in breast cancer cell lines.
- To determine the mechanism(s) of action of these analogues and to identify any particular relationships between breast cancer phenotypes and drug activity.

Chapter 2: Materials and Methods

This chapter details the materials and methods for the experimental work carried out during this thesis. At the start of each experimental chapter there is also a brief overview of the methods used.

2.1 Materials

2.1.1 General Reagents

All general laboratory reagents and plastic/glassware were from Sigma (Poole, UK) or Fisher Scientific (Loughborough, UK) and were analytical or molecular biology grade.

The bicinchoninic acid (BCA) protein assay kit was from Thermo Scientific (Hampshire, UK). Bis-acrylamide, sodium dodecyl-sulfate (SDS) and ammonium persulphate were from BioRad (Hemel Hempstead, UK). Nitrocellulose membrane was from GE Healthcare (Buckinghamshire, UK). Western blot cassettes were from Invitrogen (Paisley, UK). Blotto was made using full fat Marvel milk powder.

2.1.2 Cell Culture

RPMI 1640 (with and without phenol red), 0.025% trypsin/EDTA, phosphate buffered saline (PBS) and L-glutamine came from Invitrogen (Paisley, UK). Foetal calf serum was from Hyclone (Thermo Scientific, Hampshire, UK).

All plasticware including plates, culture flasks, centrifuge tubes and stripettes came from Corning (Fisher Scientific, Loughborough, UK).

The MTS reagent was purchased from Promega (Hampshire, UK). Isoton and accuvettes were purchase from Beckman Coulter (High Wycombe, UK).

2.1.3 Drugs

All drugs were obtained from Tocris Bioscience (Bristol, UK) apart from the following, adenosine, 2'-deoxyadenosine, 2'-deoxycytidine, inosine, hydroxyurea and 5'-amino-5'-deoxyadenosine were from Sigma (Poole,

UK). Adenosine deaminase was from Roche Diagnostics (Lewes, UK). Sterile DMSO used as the vehicle was from Sigma (Poole, UK).

2.1.4 Antibodies

The β-actin antibody was from Sigma (Poole, UK). GAPDH, cN-II, p53R2, total ERK 1/2 (tERK1/2), phosphorylated ERK 1/2 (pERK1/2), adenosine A_1 and A_{2B} receptor antibodies were all from Abcam (Cambridge, UK). The adenosine A_{2A} and A_3 receptor antibodies were from Alpha Diagnostics International (Autogen Bioclear, Nottingham, UK).

2.1.5 Annexin V/PI Flow Cytometry

The annexin V/PI apoptosis assay kit was from Abcam (Cambridge, UK). FACS flow, rinse and clean solutions were all from Becton Dickenson (Oxford, UK). FACS tubes were from (Fisher Scientific, Loughborough, UK).

2.1.6 PCR

TRI® total RNA extraction reagent, 1-bromo-3-chloropropane and isopropanol (molecular biology grade) were bought from Sigma (Poole, UK). The ImProm-II Reverse Transcription kit, 100bp DNA ladder and 6x sample loading dye were all from Promega (Hampshire, UK). The LightCycler DNA Master SYBR Green I kit and LightCycler capillaries were obtained from Roche Diagnostics (Lewes, UK). All primers were purchased from Invitrogen (Paisley, UK).

2.2 Cell Culture

2.2.1 General Cell Culture and Cell Maintenance

MCF-7 and MDAMB231 human breast adenocarcinoma cells were purchased from the European Cell Culture Collection (ECACC, UK). The human B-cell promyelocytic leukaemia cell line, HL60, was a gift from Dr. Arwyn Jones (Welsh School of Pharmacy, Cardiff, UK). The human breast cancer cell lines MDAMB436, SKBR-3, BT 474 and

T47D were a gift from The Tenovus Centre for Cancer Research (Welsh School of Pharmacy, Cardiff, UK).

All cells were routinely grown in RPMI 1640 supplemented with 4 mM L-glutamine and 10% foetal bovine serum. All cells were maintained at 37°C with 5% CO_2 in air in a humidified atmosphere and media was replenished every 3-4 days. Adherent breast cancer cells were routinely passaged when they reached ~70% confluency at a ratio of between 1:5 and 1:15. HL60 cells were split every 5-6 days between 1:15 and 1:25 dilutions

To passage adherent cells, except for the MDAMB436 cells, the media was removed and replaced with 0.025% trypsin/EDTA. Flasks were returned to the incubator and left for approximately 5 minutes until the cells detached. A two-fold greater volume of media was added to the flasks and cells were resuspended by pipetting up and down, placed into centrifuge tubes and spun at 1000 x g for 5 minutes to pellet the cells. The supernatant was poured off, and the pellet resuspended in an appropriate volume of media which was then diluted appropriately into fresh flasks and new media added. The cells were then allowed to grow as normal at 37°C.

MDAMB436 cells were passaged by scraping off the cells into new media, followed by appropriate dilutions into fresh flasks and new media being added. The cells were then allowed to grow as normal at 37°C.

To passage the HL60 cells, the cells were transferred to a centrifuge tube and centrifuged at 1000 x g for 5 minutes to pellet the cells. The supernatant was poured away, and the remaining pellet resuspended in media by repeated pipetting up and down. Appropriate dilutions were then transferred to fresh flasks, new media added and allowed to grow as normal.

Cells were not allowed to go past 15 passages from cell resuscitation in order to preserve the integrity of the cell lines and subsequent experiments.

2.2.2 Setting up 96 Well Plates

Cells were passaged in log growth phase at ~70% confluency. After collection of the cells by the appropriate methods described above, 100 µL of the cell suspension was added to 9.9 mL of Isoton (Beckman Coulter) in an accuvette and 500 µL counted in a Beckman Z2 Coulter Counter set to read particle sizes between 9 and 30 µm. The cell suspension was then diluted accordingly, and cells were seeded into 96 well plates at 5000 cells per well. No cells were seeded into edge wells to exclude possible edge effects during the assay. The cells were left to plate down for 24 hours before drug treatments were added.

2.2.3 Setting up 24 Well Plates

Cells were collected and counted as described above, and seeded into 24 well plates at a density of 10,000 cells per well. The cells were left to plate down for 24 hours before the media was changed and cells were allowed to grow for the desired time before counting

2.2.4 Setting up 6 Well Plates

As before, cells were collected, counted and seeded into 6 well plates at 300,000 cells per well for the MAPK and adenosine agonist assay, or 100,000 cells per well for drug treatment and lysis for Western blotting. In each case, the cells were then left to plate down for 24 hours before treatments or media were added.

2.2.5 Cellular Quiescence

For the MAPK/adenosine receptor agonist assay, cells were quiesced prior to addition of drug treatments for 24 hours to reduce basal MAPK signalling. After the cells had been left to plate down for 24 hours, the media was removed and cells were washed with sterile PBS to remove any residual media. The media was then replaced with quiescence

media (RPMI 1640 without phenol red, 4 mM L-glutamine and 0.1% charcoal-stripped foetal bovine serum). This media was then left on for 24 hours before the assay prior to the addition of adenosine receptor agonists and antagonists.

2.3 MTS Cell Viability Assay

To determine the cytotoxic effects and mechanism(s) of action of the various drugs, the CellTiter 96® AQ_{ueous} Assay (Promega, UK) was performed following incubation of the cells with these agents. The assay uses a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS) and the electron-coupling reagent, phenazine methosulfate. In metabolically active cells, MTS is reduced by dehydrogenase enzymes into a formazan product that is soluble in culture medium. The absorbance of the formazan is then measured at 492nm. The assay works on the principle that the quantity of the formazan product is directly proportional to the number of viable cells.

2.3.1 Setting up Treatments

Cells were set up in 96 well plates as described (2.2.2). Drug treatments at the appropriate concentrations were made up in normal growth media and warmed to 37°C. The media on the cells was then aspirated and replaced with 100 µL of drug treatment. Drug treatments and controls were repeated in at least triplicate wells and three empty wells on the edge of the plate had 100 µL of normal drug-free media added for the background control. Cells were then left for 96 hours (unless stated otherwise) at 37°C in a 5% CO_2 humidified atmosphere.

2.3.2 MTS Assay

After the required incubation time with the drug treatments the MTS assay was performed. To each well, including the three blank media control wells with no cells, 20 µL of MTS reagent was added, the plate wrapped in foil and placed back into the incubator. This was then left

for 2 ½ hours before the absorbance was determined using a microtitre plate reader (Tecan, Switzerland) at 490 nm.

The % of cell viability was then calculated by the following formula:

$$\frac{\text{(mean Abs. of treated cells – mean Abs. of blanks)}}{\text{(mean Abs. of control cells – mean Abs. of blanks)}} \times 100 = \%\ \text{cell viability}$$

Abs. (Absorbance reading)

2.4 Annexin V/Propidium Iodide Apoptosis Assay

The annexin V/propidium iodide (PI) apoptosis assay was used to determine and quantify apoptotic and dead cells after drug treatment. The assay works on the principle that one of the earliest stages of apoptosis is the translocation of phosphatidylserine residues to the outer side of the plasma membrane. Annexin V is an anticoagulant protein that preferentially binds to phosphatidylserine and, when labelled with a fluorochrome such as fluorescein isothiocyanate (FITC), can be detected by flow cytometry (Koopman *et al.* 1994). PI is then used as an indicator of plasma membrane integrity as only cells that have lost membrane integrity i.e. late apoptotic/necrotic cells will allow entry of PI and thus binding to nucleic acids.

2.4.1 Setting up Flasks

For the assay, 500,000 cells were seeded into 25 cm^2 flasks and left to plate down overnight at 37°C. Appropriate drug treatments, including positive (2 μM staurosporin), vehicle and negative controls were made up in normal growth media and warmed to 37°C. The media was then removed from the flask, and replaced with 3 mL of drug media. Positive controls were replaced with normal growth media, and then this was replaced with staurosporin-containing media 48 hours before the end of the assay. This was because staurosporin induced complete cell death

after 96 hours and the cells could not be analysed using flow cytometry.

2.4.2 Sample Preparation

After 96 hours of drug treatment, the media was removed and centrifuged at 1000 x g for 5 minutes to pellet any detached cells. The flasks were rinsed gently in PBS, and 1 mL of pre-warmed trypsin was added and left until the cells became detached. 2 mL of normal growth media was added to this, and the cell suspension was centrifuged at 1000 x g for 5 minutes. The supernatant was then removed and the cell pellet, resuspended in 1 mL of PBS, was added to the media cell pellet and resuspended by gentle pipetting up and down. This was then centrifuged again as before, and 1 mL PBS added and cells resuspended. Again this was centrifuged as before, and the PBS then removed. The cell pellet was resuspended in 500 µL annexin V binding buffer (Abcam, UK) and then passed through a fine gauge needle (21G) to create a single cell suspension. To this, 0.25 µg annexin V-FITC conjugate and 1 µg PI was added and pipetted up and down to mix. This suspension was then left on ice for 5 minutes in the dark before being analysed immediately using FACS.

2.4.3 Flow Cytometry

A BD FACSCalibur system was used with the FL1 and FL2 channels to detect FITC and PI, respectively, set to count 10,000 counts. Negative control cells (no staining) were used first to calibrate the flow cytometer using forward and side scatter to gate out debris and non-specific counts by altering the voltage, amp gain and threshold values. The region was gated and set to record counts only within this region. The voltage on the FL1 and FL2 channels was altered to bring the negative control within the first log on each scale. Next, single colour controls were used for FITC and PI to correct for colour compensation where FITC and PI channels bleed into one another by adjusting the compensation between FL1-%FL2 and FL2-%FL1. Once calibrated,

samples were analysed using a dot plot and quadrants (see representative dot plot; Figure 2.1). The data were collected using CellQuest software (Becton Dickinson, Oxford, UK) and analysed in Cyflogic software (CyFlo Ltd, Turku, Finland).

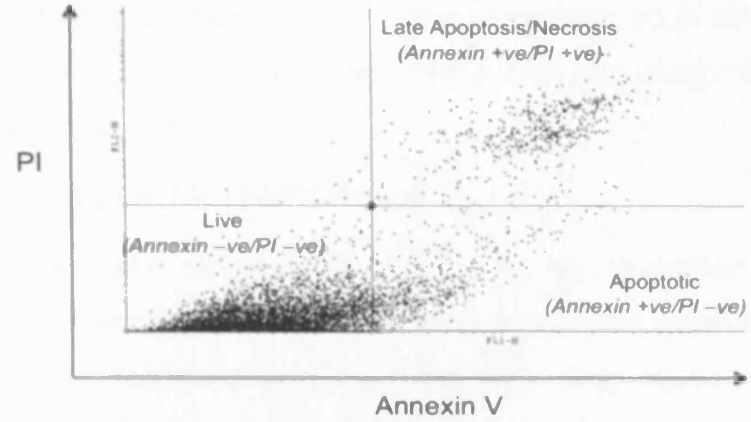


Figure 2.1 Representative dot plot obtained from Annexin V/PI stained cells and analysed with flow cytometry

Live cells that are negative for both annexin V and PI appear in the lower left quadrant. Cells in the lower right quadrant are positive for annexin V but have not taken up PI and in the early stages of apoptosis. The upper right quadrant contains cells that are in late apoptosis/necrosis and are stained with both annexin V and PI.

2.5 Western Blotting

Western blotting was used to determine the expression of proteins in cells and, combined with densitometry, was used to semi-quantify this expression. Western blotting uses antibodies to bind to and 'label' a specific protein which is then detected, in this instance using enhanced chemiluminescence (ECL).

2.5.1 Normal Cell Lysis

Cells were set up in 6 well plates as described (2.2.4) at the required seeding density. After the appropriate treatment time, media was removed and wells were gently washed in 1 x PBS twice. Lysis buffer

was then added (Tris 50 mM, EGTA 5 mM, NaCl 150 mM, Triton-X 1%, $NaVO_4$ 0.4 mM, NaF 50 mM, phenylmethanesulfonylfluoride 1 mM, phenylarsine oxide 20 mM, sodium molybdate 20 mM, leupeptin 0.1 mg/mL and aprotinin 0.1 mg/mL) to the cells, and placed upon a rocker on ice for 15 minutes. Cells were then scraped off and transferred to microcentrifuge tubes on ice. These were then spun at 16,000 x g for 15 minutes at 4°C. Supernatants were then aliquoted into boiling tubes and stored at -20°C.

2.5.2 Cell Lysis for MAPK Assay

The cells were quiesced for 24 hours as described (2.2.5). Drug treatments were made up in quiescing media and warmed to 37°C. The media was removed and replaced with drug media for the stated amount of time (0 minute time point was where the drug was added and taken off again immediately). The reaction was then stopped by removal of the drug media, and addition of the lysis buffer. Cells were then lysed as before (2.5.1).

2.5.3 Protein Assay

The BCA protein assay is a colourmetric method for determining total protein concentration in samples. It is based upon the reduction of copper ions by proteins in alkaline medium and detection of the cuprous cation. The purple-coloured reaction is formed by chelation of two molecules of BCA with one cuprous ion that exhibits strong absorbance at 592 nm and is linear with increasing protein concentrations up to ~2 mg/mL. Unknown concentrations are calculated by comparing to a standard curve of serial diluted protein concentrations.

Firstly, a serial dilution of bovine serum albumin was prepared in the range of 0.01 to 2 mg/mL in H_20. Both H_20 and lysis buffer were also used and served as background controls. The working reagent was prepared according to the manufacturer's instructions (Pierce, UK) and between 1 - 5 µL of each standard or sample was added to a 96 well

plate in duplicate. To each standard or sample, 200 µL of the working reagent was added and the plate mixed on a plate shaker for 30 seconds. The plate was then incubated for 30 minutes at 37°C before being cooled to room temperature for 5 minutes to stop the reaction.

The absorbance was then read on a microplate reader at 540 nm and the average of the duplicates for each sample or standard was calculated, with the average of the relevant blank subtracted from each calculated average. A standard curve was drawn and the concentrations of the unknown samples were calculated.

2.5.4 SDS-PAGE

Proteins were resolved routinely on 8-10% bis-acrylamide gels set in Novex gel cassettes (Invitrogen, UK). An 8-10% resolving gel was prepared (8-10% acrylamide, 375 mM Tris/HCl, 0.1% w/v SDS, 0.05% w/v ammonium persulfate and 0.05% TEMED) and poured into the cassettes. Water-saturated 2-butanol was overlaid on the gel and it was left to set for ~30 minutes. Once set, the 2-butanol was decanted and the cassette/gel was washed with distilled H_20. A 5% stacking gel was prepared (5% acrylamide, 1% w/v SDS/125 mM Tris/HCl, 0.05% w/v ammonium persulfate and 0.08% TEMED) and poured over the resolving gel immediately prior to inserting a well comb to create lanes. The gel was then left to set for 30 minutes at room temperature. Once set, the comb was removed and the gel washed with 1 x tank buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.6).

Protein samples were made up in Laemmli sample buffer at a ratio of 2:1 (6% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 125 mM Tris HCL, pH 6.8), vortexed thoroughly and then spun down. This was then heated at 95°C for 5 minutes before use. Between 5 and 20 µg of protein was loaded per well depending on the assay. A protein marker (Precision Plus Protein Standard, BioRad, Hertfordshire, UK) was added to each gel to allow

for sizing of the bands. The gels were run for 30 minutes at 50 V, followed by 120 minutes at 150 V.

2.5.5 Immunoblotting

After electrophoresis, gels were removed from the cassettes and placed into transfer buffer (42.9 mM Tris, 38.9 mM glycine, 0.038% w/v SDS and 20% methanol). Filter papers and 0.2 µm Hybond™ ECL™ nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) were cut into 6 x 8 cm pieces, the same size as the gel, and soaked in transfer buffer. The proteins were transferred from the gel to the nitrocellulose membrane using a semi-dry Multiphor II Electrophoresis system (GE Healthcare, Buckinghamshire, UK). The cathode was wetted with transfer buffer, and a stack of filter papers placed upon it. The nitrocellulose membrane was placed on top of this, followed by the gel. Another stack of filter papers were placed upon this, and any trapped air was removed by gently rolling the stack with a glass rod. Finally the anode was fitted on top of this, and gels blotted for 60 minutes at 0.8 mA/cm^2 gel.

2.5.6 Immunodetection

Once blotted, the nitrocellulose membranes were washed in 1 x TBST (50 mM Tris HCL, 150 mM NaCl, pH 7.4 and 0.1% Tween 20) and were then blocked in 5% Blotto (1 x TBST and 5% non-fat milk powder, filtered through qualitative filter paper to remove aggregates of milk powder) for at least 60 minutes at room temperature. If blocked overnight, then this was done at 4°C. Primary antibodies were diluted in 1% or 2% Blotto (1 x TBST and 1 or 2% blotto) to a final volume of 1 or 2 mL and the membranes incubated on a roller overnight at 4°C, or for 1 hour at room temperature. The next day, the blots were washed rapidly in 1 x TBST twice to remove excess antibody, followed by 2 x 5 minutes and 1 x 10 minute washes. The horseradish peroxidase-conjugated secondary antibody (range between 1:10,000 and 1:60,000) to the primary was diluted again in 1% Blotto and incubated

with the blots for 1 hour at room temperature. The blots were then washed as before, with two rapid washes followed by 2 x 5 minute and 1 x 10 minute washes.

Proteins were visualised by an ECL detection system. Excess liquid was shaken off the blots and they were placed onto Saran™ wrap. A 1:1 solution of solution A and B from the Supersignal® West Dura Extended chemiluminescent substrate kit (Pierce Biotechnology, UK) was allowed to incubated with the blots for 5 minutes. Membranes were then exposed to ECL Hyperfilm (GE Healthcare, Buckinghamshire, UK) in a dark room. Exposures ranged from 1 second to 120 minutes depending upon signal strength. If no bands appeared after this time, then exposures were left overnight to confirm no signal was present. The films were developed using Photosol RG X-ray developer and fixer (Photon Imaging Systems, UK) and the films scanned into the computer.

Densitometry was carried out on scanned images using ImageJ software (NIH, USA). Protein bands were sized by calculating an Rf value against the protein ladder standards and were expressed as the average of at least three separate blots ± S.E.M.

2.6 Real Time-PCR

Real time – polymerase chain reaction (RT-PCR) was used to semi-quantitate levels of RNA of particular genes. RT-PCR involves reverse transcribing RNA extracted from cells using a reverse-transcriptase enzyme to produce a cDNA template. This is then incubated with gene-specific primers and DNA polymerase to amplify a particular segment of DNA within the gene region by heating and cooling cycles. The end result is an exponential increase in DNA copies of this segment that can be recorded. The product can be run through an agarose gel and compared to a specific base pair sized ladder to determine the product size. In the SYBR green assay, however, SYBR green is added to the reaction mix, which preferentially binds to double-stranded DNA over

single stranded DNA, and emits a fluorescence signal. This signal is then recorded, and is proportional to the amount of gene product made. This process can be made quantitative by producing a standard curve of known RNA copy numbers of the particular gene of interest. It is semi-quantitative when making relative quantification methods against a control cell line or treatment.

2.6.1 Primer Design

Primers were designed for specific use in SYBR green real-time PCR with Beacon Designer 7.60 software (Premier Biosoft, California, USA) and checked for selectivity using Basic Local Alignment Search Tool (NCBI) and are shown in Table 2.1 below.

Gene	Primer	Sequence	Product Size	Melting Tm of Product (°C)
GAPDH	Forward	GTCGGAGTCAACGGATTT	220	81.6
	Reverse	CCTGGAAGATGGTGATGG		
dCK	Forward	CGCCACAAGACTAAGGAA	139	82.2
	Reverse	AGGATATTCACAAATGTTGACTTC		
cN-II	Forward	GACAGCAGTAGCAATGAG	195	81.2
	Reverse	GTAATACAGCAGGTTGATGA		
RRM1	Forward	ACATCCACATTGCTGAGCCTAA	195	78.5
	Reverse	TGTTCCTCTCCTTCTCTTCTTCCT		
RRM2	Forward	CTGGCTGGCTGTGACTTA	120	79.8
	Reverse	ACTGGCTGTGCTGGTTAA		
p53R2	Forward	CTTTGTTTCCCACCATTTCCCTTT	168	76
	Reverse	CTCTCCAGCCTTCCATCTTCAC		

Table 2.1 Description of Primers, Their Amplicon Sizes and Melting Temperatures.

Primers were designed to have an optimal annealing temperature of 60±2°C, be between 18-25 base pairs in length and be exon spanning if possible. Primers were selected according to these criteria and as judged by the designer software and also the likelihood of forming cross-dimers, self-dimers or hairpins.

2.6.2 RNA Extraction and Quantification

All work for PCR was done using RNase/DNase free sterile pipette tips and microcentrifuge tubes. All reagents were of molecular biology grade and free of RNase/DNase and contaminating RNA/DNA. All work was carried out on ice unless stated otherwise.

The RNA extraction procedure is based upon the widely used methods from Chomczynski and Sacchi (1987) using TRI® reagent, a total RNA isolation reagent. The reagent contains guanidine thiocyanate and phenol which act to dissolve DNA, RNA and proteins. Adherent cells were grown in a 25 cm^2 flask until ~60% confluent. The media was aspirated and 2.5 mL of TRI® reagent was added to the cells. HL60 cells were grown for 3 days after passage to ensure they were in logarithmic growth phase, spun down and 2.5 mL of TRI® reagent was added to the cell pellet and resuspended by pipetting up and down. This suspension was left on the cells for ~15 seconds and then transferred into microcentrifuge tubes. The cell suspensions were centrifuged at 12,000 x g for 10 minutes at 4°C to remove cell debris. The supernatant was then transferred into microcentrifuge tubes. In a fumehood, 0.5 mL of 1-bromo-3-chloropropane was added, tubes were capped and shaken vigorously for 15 seconds before being left for 15 minutes at room temperature. This mixture was then aliquoted equally into two microcentrifuge tubes and centrifuged at 12,000 x g for 15 minutes at 4°C. The upper, clear aqueous phase containing the RNA was removed into a sterile tube and 1.5 mL 2-propanol was added and vortexed. This mixture was allowed to stand at room temperature for 10 minutes before being centrifuged again at 12,000 x g for 15 minutes at

4°C. The supernatant was carefully removed, being careful not to disturb the RNA pellet at the bottom of the tube, before being allowed to air dry at room temperature. The pellet was then washed by adding 2.5 mL of 75% aqueous ethanol, and pipetted up and down. The sample was then vortexed and centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol was then removed and the pellet allowed to nearly dry, being careful not to over-dry the pellet. The pellet was then suspended in PCR-grade water and stored at -80°C until use.

RNA content and purity was established by determining the optical density at 260 nm and 280nm by UV spectroscopy. Absorbance at 260 nm measures RNA content whereas absorbance at 280 nm measures protein content. RNA content compared to protein content is used to establish the purity and yield of RNA from the extraction and a ratio of ≥1.7-2.0 RNA to protein is considered suitable. Total RNA yield and concentration is calculated from the following formulae:

Total A_{260} nm = optical density x dilution factor

*RNA Concentration (µg/mL) = Total A_{260} nm x 40**

RNA Yield = RNA concentration x Original resuspension volume

** 1 optical density unit of single-strand RNA is = 40 µg/mL*

2.6.3 Reverse Transcription

The Im-Prom II Reverse Transcription kit (Promega, USA) was used for the reverse transcription and the method was followed as described by the manufacturer. To 1 µg RNA, 0.5 µg of Oligo(dT)15 was added and made to volume (10 µL) with H_20. The solution was heated at 70°C for 5 minutes and then rapidly quenched on ice for at least 5 minutes. Tubes were spun down to collect any condensate. To this, 5 x reaction buffer, $MgCl_2$ (3.25 mM final concentration), dNTPs (0.5 mM final concentration for each dNTP), RNase inhibitor (20 IU/per reaction) and reverse transcriptase (1 IU/per reaction) were added, made up to

volume (20 µL) with H_20 and mixed by pipetting up and down. This solution was then incubated at 25°C for 5 minutes followed immediately by 42°C for 1 hour. The reverse transcriptase was then inactivated by heating for 15 minutes at 70°C. cDNA were then stored at -80°C until use.

2.6.4 RT-PCR

The RT-PCR was carried out using the LightCycler® DNA Master SYBR Green I kit (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's instructions. A master mix was prepared containing H_20, $MgCl_2$ (3 mM final concentration), primers (0.5 µM final concentration each) and 1 x SYBR green I mix which contains Taq DNA polymerase, dNTP mix, SYBR Green I dye and 10 mM $MgCl_2$, which was made to a final volume of 19 µL per reaction and scaled up accordingly. To each capillary, 19 µL of the master mix was added, and 1 µL of cDNA or 1 µL of the reverse transcription negative control (RNA that was incubated under the same conditions as normal, but the reverse transcriptase enzyme was replaced with H_20) or H_20 control was added. These were capped and pulsed in a microcentrifuge to transfer the liquid from the reservoir into the capillary body. The capillaries were then transferred to the LightCycler and the PCR was run immediately.

The conditions for the PCR runs are detailed in the methods section of each chapter accordingly. All PCR runs followed the same protocol:

1. Denaturation – initial separation of the DNA strands
2. Amplification
    a. Denaturation – DNA strand separation
    b. Annealing – primer annealing
    c. Elongation – elongation of the product
3. Melting Curve Analysis – increased heating for amplicon identification

4. Cooling – cools the rotor and samples chamber

The exception to this was when PCR amplicons were to be run under gel electrophoresis for size comparison where the melting curve analysis step was omitted.

2.6.5 Gel Electrophoresis

For amplicon size analysis, PCR amplicons were run on a 2% agarose gel to separate the amplicons based upon size and compared to a base pair ladder of known sizes.

After the cooling step, the capillaries were de-capped, and place upside down into DNase/RNase free 1.5 mL microcentrifuge tubes and pulsed in a microcentrifuge for 5 seconds to transfer the sample into the tube. These were then capped and stored at -20°C until use, but were not kept for more than 48 hours. A 2% agarose gel was prepared in 1 x Tris acetate EDTA (TAE) buffer and contained 0.005% ethidium bromide. Samples were mixed with loading dye and 15 µL was loaded into the gel. The gel was then run at 100V for approximately 1 hour. The gel was then visualised under UV illumination and photographed using an Alpha DigiDoc room temperature system (Alpha Innotech, USA).

2.6.6 Relative Quantification

Relative quantification of RNA to a particular gene measures the relative difference in RNA levels compared to a control treatment, sample or group. It does not determine an absolute copy number, as can only be achieved with a standard curve (Pfaffl *et al.* 2006). Relative quantification was used for this thesis due to the lack of availability of standards for the different genes investigated and also as this method allows for comparisons between multiple PCR runs (Pfaffl *et al.* 2006).

To determine expression, the threshold cycle (*Ct*) value is calculated by the LightCycler Software v3.5 (Roche, Burgess Hill, UK) using the second derivative maximum method. The *Ct* value is the number of cycles required for the fluorescent signal to pass a predetermined

threshold i.e. the more RNA, the lower the *Ct* value will be. This method calculates the *Ct* value automatically and therefore reduces user subjectivity and increases reproducibility.

Relative quantification of the genes was determined using the HL60 cell line as the control group comparator. Each PCR run included a negative control as described (2.6.4) and GAPDH as a positive control. All data were then normalised to GAPDH data to correct for any differences in starting RNA and cDNA levels. The normalised data were uploaded into Relative Expression Software Tool (REST, Germany) and the relative expression of genes was calculated compared to HL60 cells. The software determines significant differences between groups using a pair-wise fixed reallocation randomisation test (Pfaffl *et al.* 2002).

2.7 Cell Growth Assay

Cells were seeded into 24 well plates as described (2.2.3), set up in triplicate. After being left to plate down overnight, the media was aspirated and replaced with 1 mL fresh media. The cells were then returned to the incubator and left for the required time, 24, 48 or 96 hours. At the appropriate time point, the media was removed from the cells, and replaced with 0.1% trypsin/EDTA and returned to the incubator until the cells were detached. The cells were taken up into a syringe through a 21G needle to create a single cell suspension. 1 mL of Isoton was added to the well to obtain any remaining cells, and this was also taken up into the syringe. This step was repeated twice more until a final volume of 4 mL was in the syringe. This was then transferred into an Accuvette containing 6 mL of Isoton and counted on the Beckman Coulter Counter Z2 set to measure 500 µL reading particles between 9 – 30 µm. Readings from each Accuvette were taken in duplicate and the mean taken.

2.8 Data Analysis

All IC_{50} data were calculated using GraphPad Prism v5.0b for Mac OS X. Concentration response curves were fitted using non-linear regression with a fixed slope (assumes a Hill slope = -1). This uses the following formula to calculate the IC_{50}:

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(X - \text{Log } IC_{50})}}$$

Where:

y = response
x = log (drug concentration)
Top = y value at the top of the plateau
Bottom = y value at the bottom of the plateau

IC_{50} values are presented as the mean and the 95% confidence intervals (C.I.). Comparisons between IC_{50} of different cell lines were determined in GraphPad Prism, using either a Student's *t* test for two groups, or a one-way analysis of variance (ANOVA) with a Tukey-Kramer *post hoc* test to compare multiple groups. Probability values (p) of less than 0.05 were considered statistically significant.

The cell MTS (excluding the dose-response curves explained above), annexin V FACS and densitometry data from Western blotting are displayed as the mean ± S.E.M. unless otherwise stated. Comparisons between groups were determined in GraphPad Prism, using either a Student's *t* test for two groups, or a one-way ANOVA with a Tukey-Kramer *post hoc* test if a significant difference was observed between three or more groups. If only selected means were compared than the Bonferroni correction test was used. The Kramer correction corrects for unequal sample sizes. The Tukey-Kramer assumes normally distributed data, although this was not calculated as the *n* were too small (≥3 but <5). A two-way ANOVA was used to determine statistical significance

when two independent variables were being considered, such as time and concentration, followed by a Bonferroni *post hoc* test. Probability values (p) of less than 0.05 were considered statistically significant.

Correlations between IC_{50} values and gene expression (determined by REST software, section 2.6.6) were determined using GraphPad Prism. The Pearson correlation coefficient (r) was calculated for each gene compared to the IC_{50} value. Probability values (p) of less than 0.05 were considered statistically significant.

Chapter 3: The Effects of Adenosine and 2'-Deoxyadenosine on Human Breast Cancer Cells

3.1 Introduction

Adenosine and 2'-deoxyadenosine are essential purine nucleosides that have functions in regulating blood flow, inflammation and neuronal excitability. They are also precursors for essential molecules such as cAMP, ATP and as a nucleobase in DNA/RNA (Yoon and Rothman 1991; Cronstein 1994; Davis *et al.* 2003).

Synthesis occurs primarily by dephosphorylation of adenine nucleotides or via hydrolysis of *S*-adenosylhomocysteine (Luippold *et al.* 1999; Löffler *et al.* 2007). Their metabolism is regulated primarily by ADA, which deaminates adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively (Öztürk *et al.* 2008). Cellular uptake helps regulate the extracellular concentration of these purines and occurs via ENTs and CNTs (Fang *et al.* 1996). Intracellular adenosine and 2'-deoxyadenosine can be phosphorylated by adenosine kinase and successive purine kinases to nucleotides (Lloyd and Fredholm 1994).

Adenosine concentrations are known to significantly exceed physiological levels in states of hypoxia including those observed in the extracellular fluid of solid tumours (Blay *et al.* 1997) where it is suggested to play a role in stimulating angiogenesis (Ryzhov *et al.* 2007). This increase in adenosine may also aid tumour development by functioning as an immunosuppressant (Linden 2006). Several studies have also suggested that adenosine can stimulate the growth of tumour and normal cell lines by mechanisms independent of immunosupression or angiogenesis and by both adenosine receptor-dependent and independent means (Lemmens *et al.* 1996; Mujoomdar *et al.* 2003; Jacques-Silva *et al.* 2004; Mujoomdar *et al.* 2004; Gessi *et al.* 2007).

Despite evidence highlighting the role of adenosine in supporting tumour growth, numerous studies have identified adenosine and 2'-deoxyadenosine as inhibiting the growth of human breast, colon, gastric, ovarian and leukaemic cancer cell lines mostly via a mechanism requiring intracellular phosphorylation (Tanaka *et al.* 1994; Barry and Lind 2000; Saitoh *et al.* 2004; Hashemi *et al.* 2005). Other groups have identified non-specific intracellular mechanisms of action for adenosine in hepatocellular carcinoma cells (Wu *et al.* 2006) and in U973 cells (Schneider *et al.* 2001). The clinically used anti-cancer agent deoxycoformycin is an inhibitor of ADA and is suggested to work by increasing intracellular adenosine and 2'-deoxyadenosine nucleotides causing an imbalance of dNTP pools and inhibiting *S*-adenosylhomocysteine hydrolase. These effects lead to an impaired ability of the cell to synthesize and repair DNA, ultimately leading to either cellular quiescence or cell death (Fidias *et al.* 1996; Dillman 2004). Further evidence that supports an intracellular mechanism of action comes from work with the PNA drugs cladribine, fludarabine and clofarabine that are all known to work by intracellular phosphorylation, and completely independently of adenosine receptor activation (Galmarini *et al.* 2001).

However, there is evidence that the anti-cancer activity of adenosine and adenosine agonists is due to adenosine receptor activation via A_1 (Colquhoun and Newsholme 1997; Sai *et al.* 2006), A_{2A} (Merighi *et al.* 2003) or A_3 receptors (Yao *et al.* 1997; Fishman *et al.* 2000; Ohana *et al.* 2001; Panjehpour and Karami-Tehrani 2004; Lee *et al.* 2005; Panjehpour and Karami-Tehrani 2007; Bar-Yehuda *et al.* 2008; Kim *et al.* 2008). Interestingly, a number of studies have found that adenosine receptor agonists, particularly A_3 receptor agonists such as Cl-IB-MECA and IB-MECA, are able to induce apoptosis of various cancer cell lines via a mechanism independent of the A_3 or any adenosine receptor (Lu *et al.* 2003; Cheung *et al.* 2006).

There are conflicting reports pertaining to the effects of adenosine receptor activation and the endogenous compounds adenosine and 2'-deoxyadenosine being either tumour promoters or tumour suppressors via a variety of different mechanisms. Further elucidation and confirmation of the precise roles of the receptors and adenosine and 2'-deoxyadenosine may open up novel therapeutic options for the treatment of breast cancers.

3.2 Chapter Aims

The aim of this chapter was:

- To investigate the effects of adenosine and 2'-deoxyadenosine on the proliferation of breast cancer cell lines and elucidate their mechanisms of action

The specific objectives of this chapter were:

- To establish the expression of adenosine receptors and determine their involvement in cell proliferation.
- To investigate the effects of endogenous and exogenous adenosine and 2'-deoxyadenosine on breast cancer cell viability
- To determine the mechanisms of action of adenosine and 2'-deoxyadenosine

3.3 Methods

The protocols for the MTS, Western blotting and MAPK assays were described in detail in Chapter 2.

3.3.1 MTS Assay

Cells were seeded at 5000 cells/well into 96 well plates and allowed to plate down overnight. Media was aspirated from the adherent cells and replaced with media containing combinations or single drugs or enzymes from ADA (2U/mL), inosine (100 μM), deoxycoformycin (1 μM), CGS 15943 (1 μM) or 5-amino-5'-deoxyadenosine (50 μM). Adenosine and 2'-deoxyadenosine (0.01 – 1000 μM) were used in the presence of 1 μM deoxycoformycin. Relevant media only, vehicle (DMSO 0.25%) and blank controls were included on each plate. Cells were then left for 96 hours at 37°C in 5% CO_2 in a humidified atmosphere. The MTS assay was then performed as described (section 2.3.2).

3.3.2 Western Blotting

MCF-7 and MDAMB231 cell lysates were prepared from cells in logarithmic growth phase and protein quantified as described in sections 2.5.1 and 2.5.3, respectively. Routinely, 20 μg of protein were separated using SDS-PAGE and proteins were then transferred to nitrocellulose membrane using semi-dry immunoblotting (section 2.5.5). After blocking, membranes were incubated with the primary antibodies overnight at 4°C in 1% Blotto before being washed, probed with secondary antibodies, washed again and detected using ECL. The concentrations of primary and secondary antibodies are given below.

β-actin	1°	1:50,000
	2°	1:50,000 anti-mouse
A_1	1°	1:2000

	2°	1:10,000 anti-rabbit
A_{2A}	1°	1:1000
	2°	1:10,000 anti-rabbit
A_{2B}	1°	1:1000
	2°	1:10,000 anti-rabbit
A_3	1°	1:250
	2°	1:10,000 anti-rabbit

3.3.3 MAPK Assay

MCF-7 and MDAMB231 cells were quiesced as described (see Section 2.2.5). Adenosine or 2'-deoxyadenosine (100 μM) and deoxycoformycin (1 μM) alone or in combination with the non-selective adenosine receptor antagonist CGS 15943 (1 μM) were added to the cells for the time periods indicated (0, 5, 10, 30 or 60 minutes) before being aspirated and lysis buffer added. Cells were then lysed and lysates subjected to SDS-PAGE and Western blotting as described (see Section 2.5). Controls of media only, DMSO (0.02%), CGS 15943 and deoxycoformycin alone were also included. The concentrations of primary and secondary antibodies are given below.

GAPDH	1°	1:50,000
	2°	1:50,000 anti-mouse
phospho-ERK 1/2	1°	1:2000
	2°	1:10,000 anti-rabbit
total-ERK 1/2	1°	1:1000
	2°	1:10,000 anti-rabbit

3.3.4 Data Analysis

All data are expressed as mean ± S.E.M. except for the IC_{50} data which are expressed as the mean and the 95% confidence intervals (C.I.) Data were entered into GraphPad Prism V5.0b and graphs were plotted. IC_{50} values were calculated using non-linear curve regression with a fixed Hill slope according to the following formula:

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(X - \text{Log } IC_{50})}}$$

Where:

y = response

x = log (drug concentration)

Top = y value at the top of the plateau

Bottom = y value at the bottom of the plateau

Comparisons between two groups were made using a Student's *t* test and a one-way ANOVA was used for three or more groups with a Tukey-Kramer *post hoc* test if a significant difference was observed or a Bonferroni *post hoc* test when comparing only selected pairs of data. Probability values of less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 Expression of adenosine receptors in MCF-7 and MDAMB231 cells

Western blotting was used to determine the expression of adenosine receptors in the MCF-7 and MDAMB231 cell lines. The band intensities were not quantified as only the expression of the receptors was sought.

The A_1 receptor was present in both cell lines in apparently relative equal amounts (Figure 3.1 A) and appeared as a band with an average molecular mass of 35.3±0.5 KDa compared to the reported size of 36.5 KDa.

The A_{2A} receptor was present in both cell lines, seen as a band at 43.9±1.2 KDa (expected molecular mass 44.7 KDa) (Figure 3.1 B) but appeared stronger in the MDAMB231 compared to the MCF-7 cells. Strong bands appeared consistently at ~49 and 32 KDa which appeared stronger in the MCF-7 cells compared to the MDAMB231 cells. Other faint bands of high molecular mass >50 KDa frequently appeared in lysates from both cell lines.

The expression of the A_{2B} was also equal apparently between the cell lines (Figure 3.1 C) and appeared as lone bands with a molecular mass of 37.6±0.6 KDa compared to the reported molecular mass of 36.3 KDa.

The A_3 receptor was also present in both cell lines, but with an apparently stronger signal in the MCF-7 cells (Figure 3.1 D). Other bands were present at high molecular mass >50 KDa. Bands corresponding to the expected size of A_3 receptor (36.2 KDa) appeared with a molecular mass of 35.7±0.5 KDa.

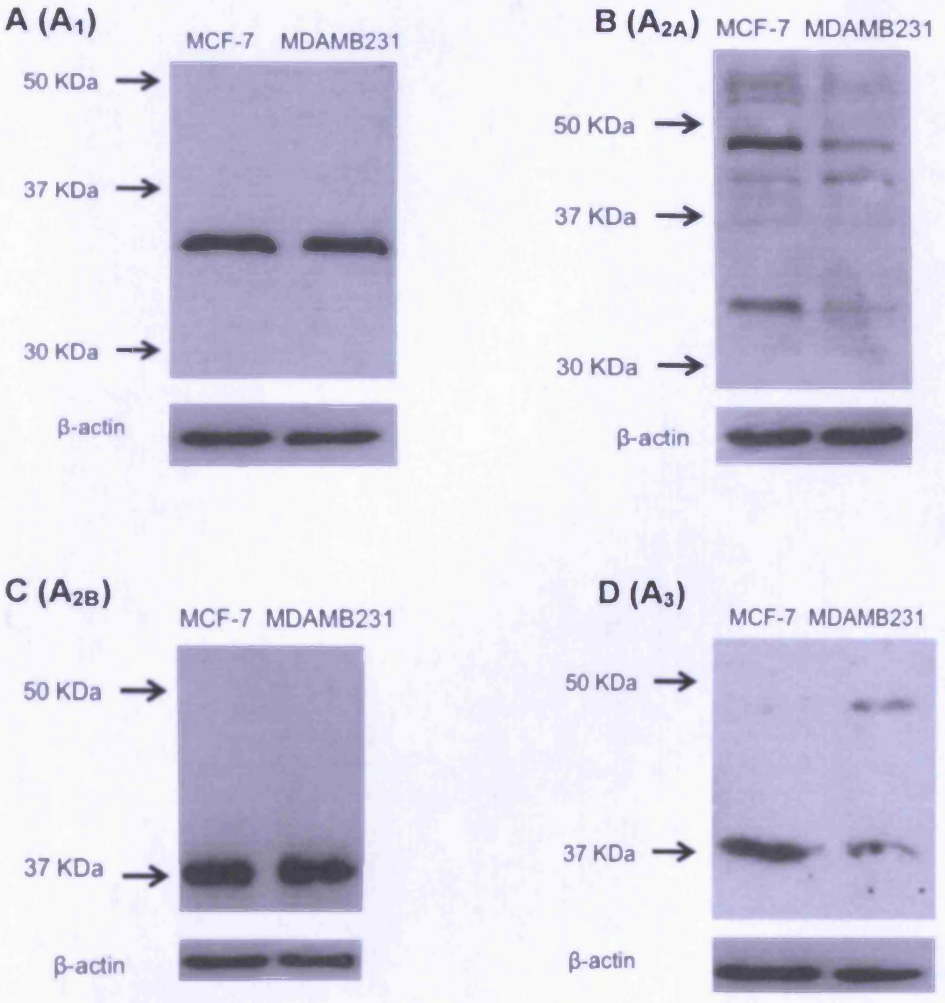


Figure 3.1 The Expression of Adenosine Receptors in MCF-7 and MDAMB231 Cells

The expression of the four adenosine receptors A_1 (A), A_{2A} (B) A_{2B} (C) and A_3 (D) were determined by Western blotting in the MCF-7 and MDAMB231 cell lines. Protein loading was confirmed by probing for β-actin. Representative blots of three separate experiments are shown. The expected molecular masses of the receptors are A_1 36.5 KDa, A_{2A} 44.7 KDa (indicated by the arrow), A_{2B} 36.3 KDa and A_3 36.2 KDa.

3.4.2 Effects of adenosine receptor antagonists on cell viability

To determine if endogenous activation of adenosine receptors was important for cell proliferation, selective receptor antagonists were used to block the receptors and cell viability was then determined with the MTS assay. The selective receptor antagonists used were SLV 320 (A_1), SCH 442416 (A_{2A}), PSB 603 (A_{2B}) and MRS 1334 (A_3) and were also used at selective concentrations for their receptors (100 nM; Klotz *et al.* 2000; Kalk *et al.* 2007).

In both cell lines, none of the selective receptor antagonists at these concentrations had any significant effect upon cell viability (Figure 3.2 A and B).

3.4.3 Effects of a non-selective adenosine receptor agonist on cell viability

Next to determine if exogenous activation of adenosine receptors could influence cell viability, the non-selective adenosine receptor agonist NECA (Klotz *et al.* 2000) was incubated with the cells and viability determined by the MTS assay.

In the MCF-7 cells, NECA significantly reduced cell viability by 16.9±2.0% and 32.0±2.4% compared to control at 30 ($p<0.05$) and 100 μM ($p<0.001$), respectively (Figure 3.3). At concentrations up to 100 μM, NECA had no significant effect upon MDAMB231 cell viability (Figure 3.3).

3.4.4 Effects of manipulating endogenous adenosine and 2'-deoxyadenosine levels on cell viability

To investigate the function of endogenous adenosine and 2'-deoxyadenosine on cell viability, various agents were used to increase or decrease their metabolism.

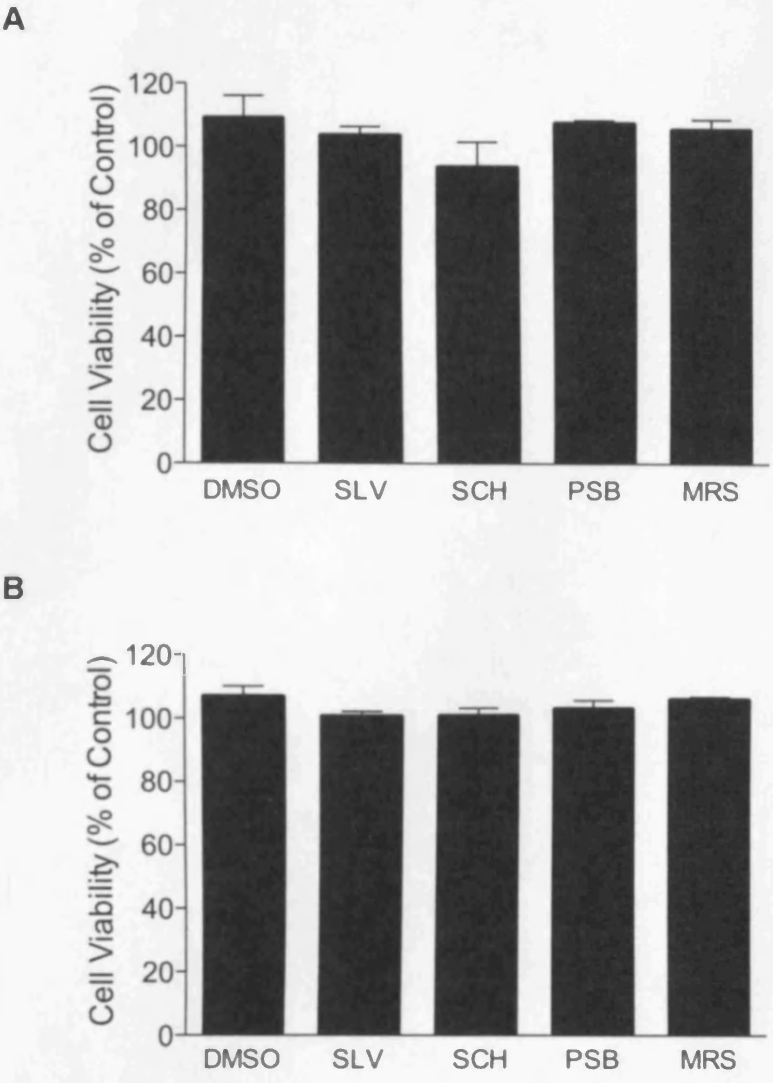


Figure 3.2 The Effects of Selective Adenosine Receptor Antagonists on Cell Viability in MCF-7 and MDAMB231 Cells

The effects of the selective adenosine receptor antagonists (100 nM) SLV302 (SLV; A_1), SCH442416 (SCH; A_{2A}), PSB603 (PSB; A_{2B}) and MRS1334 (MRS; A_3) on cell viability in MCF-7 (A) and MDAMB231 (B) cells were determined by the MTS assay after 96 hours. Data are expressed as the mean of three separate experiments repeated in triplicate ± S.E.M. Data were analysed using a one-way ANOVA and no significant differences were found.

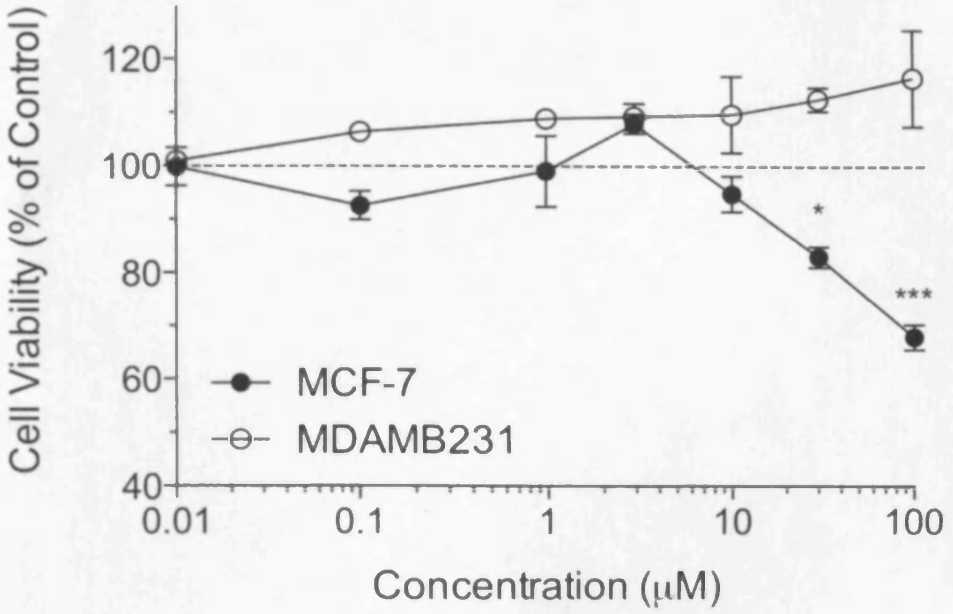


Figure 3.3 Effects of the Non-Selective Adenosine Receptor Agonist NECA on MCF-7 and MDAMB231 Cell Viability

*The effects of the non-selective adenosine receptor agonist, NECA, on cell viability were investigated in the MCF-7 and MDAMB231 cell lines after 96 hours using the MTS assay. Data are expressed as the mean of three separate experiments repeated in triplicate ± S.E.M. The dotted line indicates control (100%). Data were analysed using a one-way ANOVA and a Bonferroni post hoc test comparing selected pairs of means (between control and drug). *($p<0.05$), ***($p<0.001$) compared to control.*

ADA (2U/mL Lopes *et al.* 1999), the enzyme that metabolises adenosine and 2'-deoxyadenosine, significantly ($p<0.001$) reduced cell viability to 37.8±1.0% and 18.0±1.7% compared to control in the MCF-7 and MDAMB231 cells, respectively (Figure 3.4). There was also a significantly greater reduction ($p<0.001$) in viability of MDAMB231 cells compared to MCF-7 cells with ADA treatment.

The ADA inhibitor, deoxycoformycin (1 μM) and adenosine metabolite inosine (100 μM) had no significant effects on cell viability in either the MCF-7 or MDAMB231 cells (Figure 3.4).

3.4.5 Effects of Exogenous adenosine and 2'-deoxyadenosine on cell viability

Next, the effects of adding exogenous adenosine and 2'-deoxyadenosine on cell viability were investigated. These studies were conducted in the presence of deoxycoformycin (1 μM) to inhibit the metabolism of adenosine and 2'-deoxyadenosine by ADA.

Adenosine was able to significantly reduce the number of viable MCF-7 and MDAMB231 cells compared to control at concentrations ≥10 and ≥100 μM in the MDAMB231 and MCF-7 cells, respectively. 2'-Deoxyadenosine was able to significantly ($p<0.001$) reduce cell viability in MCF-7 and MDAMB231 cells at concentrations ≥30 μM compared to control (Figure 3.5 A and B). The IC_{50} values for adenosine in the MCF-7 and MDAMB231 cells were 139.7 μM (-90.1 – 369.4) and 17 μM (-7.7 - 41.8), respectively. The IC_{50} values for 2'-deoxyadenosine in the MCF-7 and MDAMB231 cells were 46.6 μM (22.3 – 79.8) and 58.5 μM (3.7 - 113.3), respectively. There were no significant differences in the IC_{50} values for either adenosine or 2'-deoxyadenosine compared to one another or between cell lines.

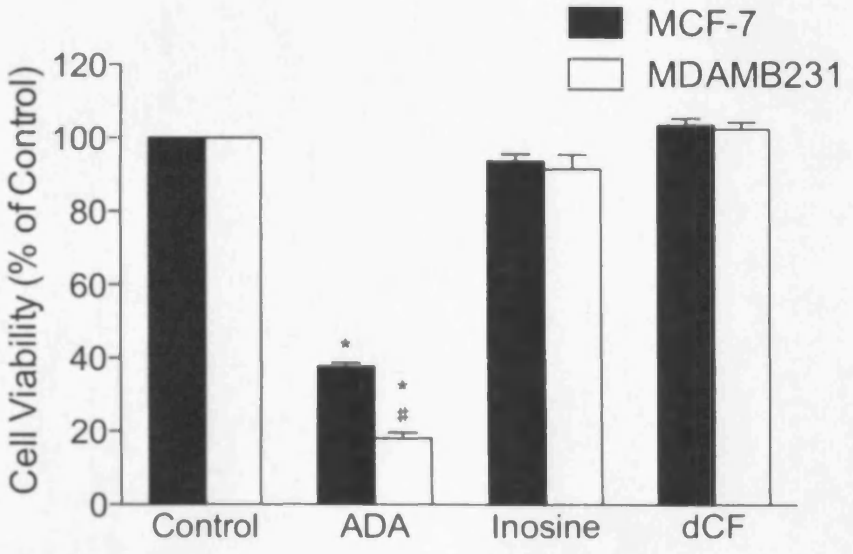


Figure 3.4 Effects of Manipulating Endogenous Adenosine and 2'-Deoxyadenosine Levels on MCF-7 and MDAMB231 Cell Viability

*The effects of manipulating endogenous adenosine and 2'-deoxyadenosine levels on MCF-7 and MDAMB231 cell viability was investigated using the MTS assay after 96 hours using adenosine deaminase (2U/mL; ADA), deoxycoformycin (1 μM; dCF) and inosine (100 μM). Data are expressed as the mean of three separate experiments repeated in triplicate ± S.E.M. Data were analysed using a one-way ANOVA and a Tukey-Kramer post hoc test. *($p<0.001$) compared to control (media only). #($p<0.001$) compared to the MCF-7 cells.*

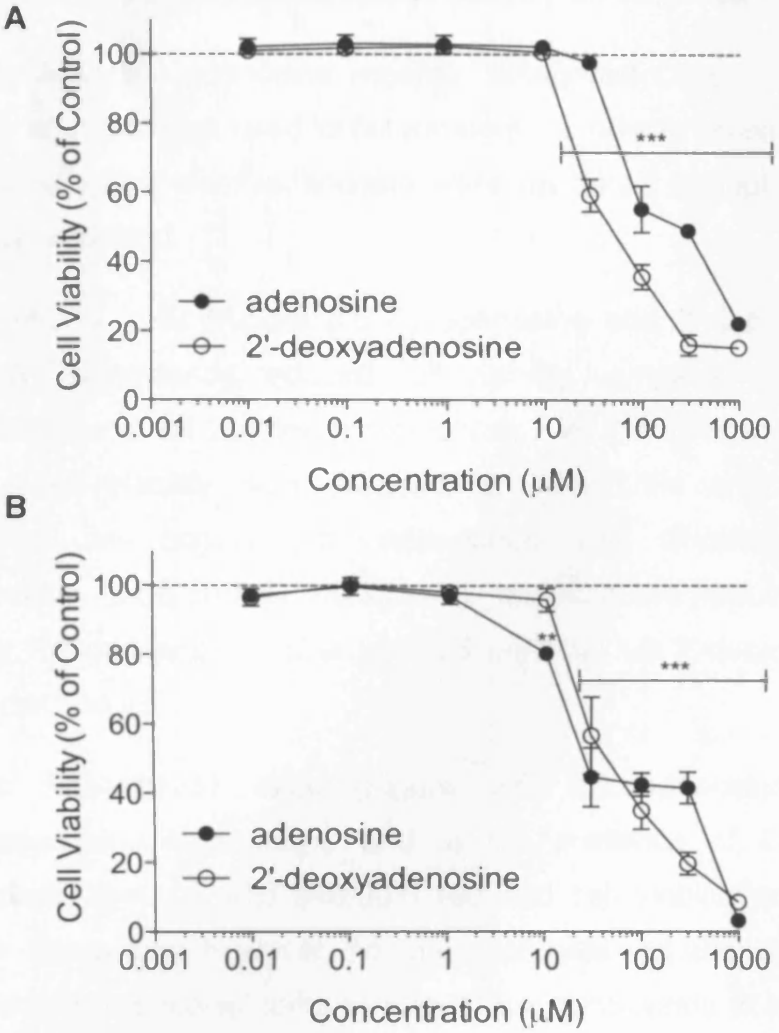


Figure 3.5 Effects of Exogenous Adenosine and 2'-Deoxyadenosine on MCF-7 and MDAMB231 Cell Viability

*The effects of exogenous adenosine and 2'-deoxyadenosine in the presence of deoxycoformycin (1 μM) on MCF-7 (A) and MDAMB231 (B) cell viability were investigated using the MTS assay after 96 hours. Data are the mean of three separate experiments repeated in triplicate ± S.E.M. The dotted line indicates control (100%). Data were analysed with a one-way ANOVA and Tukey-Kramer post hoc test. **($p<0.01$), ***($p<0.001$) compared to control.*

3.4.6 Effects of the adenosine receptor antagonist CGS 15943 on adenosine and 2'-deoxyadenosine activity on cell viability

The non-selective adenosine receptor antagonist CGS 15943 (1 μM; Klotz *et al.* 2000) was used to determine if the effects on cell viability of adenosine and 2'-deoxyadenosine were mediated through adenosine receptor activation.

In the MCF-7 cells (Figure 3.6 A) adenosine and 2'-deoxyadenosine (100 μM) significantly reduced cell viability compared to control to 52.8±4.8% and 94.2±1.5%, respectively. In the presence of CGS 15943, cell viability was reduced to 75.9±5.5% and 90.5±2.3% compared to control for adenosine and 2'-deoxyadenosine, respectively. CGS 15943 significantly inhibited the reduction in cell viability induced by adenosine ($p<0.05$, $n=3$) but not 2'-deoxyadenosine in this cell line.

In the MDAMB231 cells (Figure 3.6 B), adenosine and 2'-deoxyadenosine both alone and in the presence of CGS 15943 significantly ($p<0.05$ and $p<0.001$) reduced cell viability compared to control. There was, however, no difference between adenosine and 2'-deoxyadenosine alone compared to these compounds in combination with CGS 15943.

3.4.7 Effect of adenosine and 2'-deoxyadenosine on ERK 1/2 phosphorylation

The effects of adenosine and 2'-deoxyadenosine (100 μM; plus 1 μM deoxycoformycin) on ERK1/2 phosphorylation were investigated in MCF-7 and MDAMB231 cells alone, and in the presence of the non-selective adenosine receptor antagonist CGS 15943 (1 μM).

In the MCF-7 cells, there were relatively consistent levels of phosphorylated ERK1/2 in the media only, CGS, deoxycoformycin, 2'-deoxyadenosine and 2'-deoxyadenosine plus CGS treatment groups

over time. Treatment with adenosine (in the presence of deoxycoformycin) increased ERK1/2 phosphorylation compared to the media group from 5 minutes up to 30 minutes treatment and this effect was reduced with the addition of CGS 15943 (Figure 3.7).

In the MDAMB231 cells phosphorylation of ERK1/2 was consistent over time in the media, CGS 15943 and deoxycoformycin alone treatment groups. ERK1/2 phosphorylation decreased over time with adenosine and 2'-deoxyadenosine (in the presence of deoxycoformycin) both alone and in combination with CGS 15943 (Figure 3.7).

3.4.8 Effects of the adenosine kinase inhibitor 5'-amino'-5'-deoxyadenosine on adenosine and 2'-deoxyadenosine activity on cell viability

To examine the role of the purine phosphorylating enzyme, adenosine kinase in the actions of adenosine and 2'-deoxyadenosine on cell viability, the adenosine kinase inhibitor 5'-amino-5'-deoxyadenosine (50 μM; Barry and Lind 2000) was used.

In the MCF-7 cells, (Figure 3.8 A) adenosine and 2'-deoxyadenosine (100 μM) significantly reduced ($p<0.001$) cell viability compared to control to 50.7±0.1% and 22.73±1.6%, respectively. In the presence of the kinase inhibitor, viability was significantly reduced ($p<0.001$) to 61.8±2.9% and 71.5±5.8% compared to control for adenosine and 2'-deoxyadenosine, respectively. In both cases, the presence of the kinase inhibitor significantly reduced the action of adenosine ($p<0.01$) and 2'-deoxyadenosine ($p<0.001$) on cell viability but viability was still significantly reduced ($p<0.001$) compared to 5'-amino-5'-deoxyadenosine alone.

In the MDAMB231 cells (Figure 3.8 B), cell viability was significantly reduced when treated with adenosine ($p<0.01$) and 2'-deoxyadenosine ($p<0.001$) compared to control by 30.4±5.9% and 50.4±2.5%, respectively.

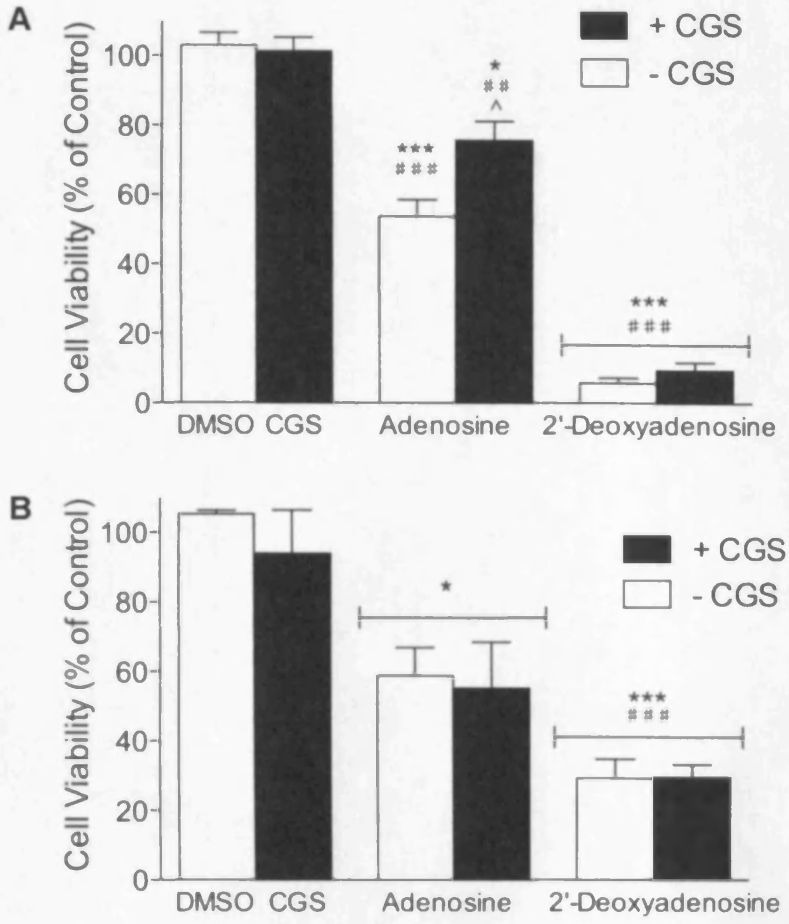


Figure 3.6 Effects of the Adenosine Receptor Antagonist CGS 15943 on Adenosine and 2'-Deoxyadenosine Activity on Cell Viability

*The effects of adenosine and 2'-deoxyadenosine (100 μM) on cell viability were investigated in the presence of the non-selective adenosine receptor antagonist, CGS 15943 (1 μM) using the MTS assay after 96 hours in MCF-7 (A) and MDAMB231 (B) cells. All adenosine and 2'-deoxyadenosine data are in the presence of deoxycoformycin (1 μM). Data are the mean of three separate experiments repeated in triplicate ± S.E.M, analysed with a one-way ANOVA and Tukey-Kramer post hoc test. *($p<0.05$), ***($p<0.001$) compared to control. ##($p<0.01$), ###($p<0.001$) compared to CGS alone. ^($p<0.05$) compared to adenosine alone.*

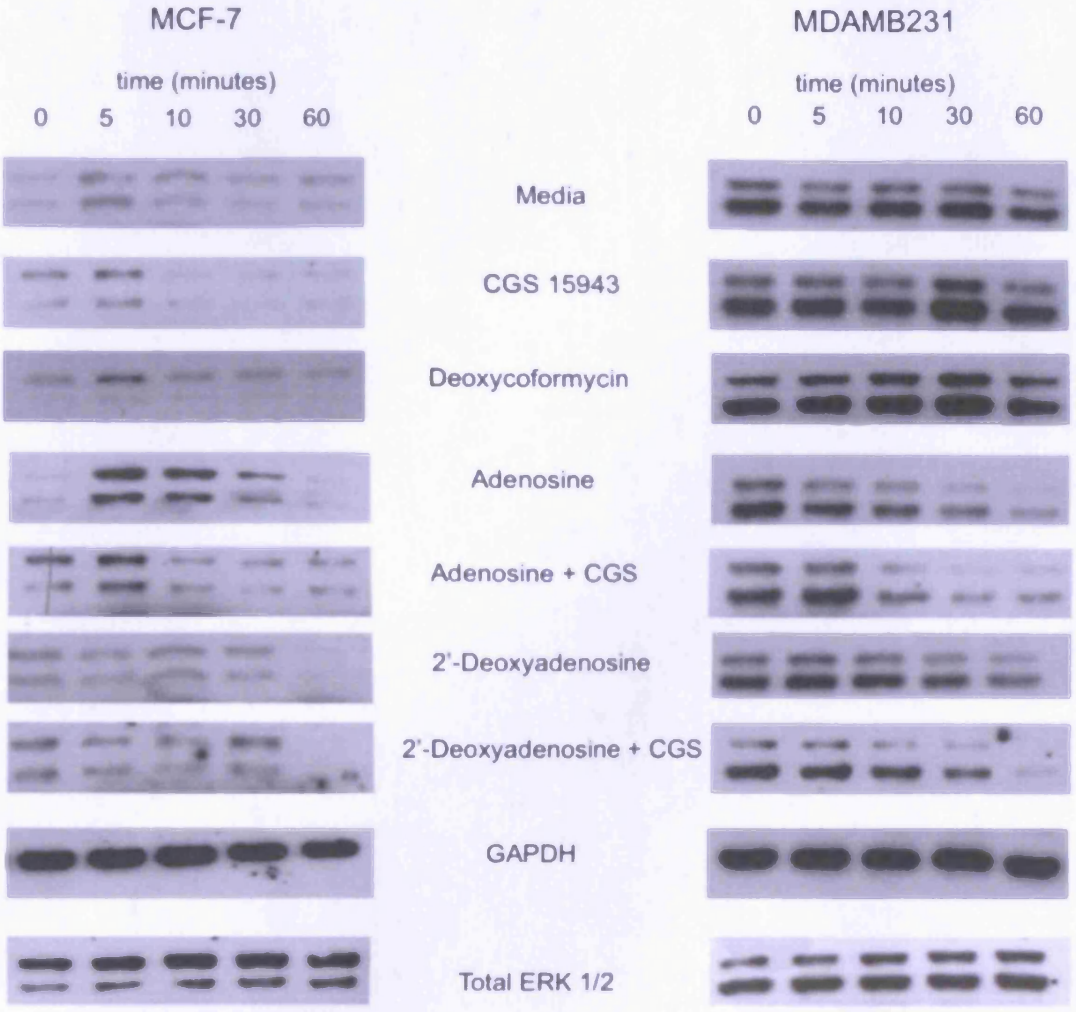


Figure 3.7 Effect of Adenosine and 2'-Deoxyadenosine on ERK 1/2 Phosphorylation in MCF-7 and MDAMB231 Cells

The effects of media alone, CGS 15943 (1 μM), deoxycoformycin (1μM), adenosine and 2'-deoxyadenosine (100μM) plus deoxycoformycin alone or in the presences of CGS 15943 on the phosphorylation of ERK 1/2 in quiesced MDAMB231 and MCF-7 cells by Western blotting. Representative blots are shown from three independent experiments. GAPDH was used to confirm protein loading and a representative blot of total ERK 1/2 is also shown.

In the presences of the kinase inhibitor, the actions of adenosine were not significantly different compared to adenosine alone. The inhibition of cell viability by 2'-deoxyadenosine was significantly reduced ($p<0.01$) in the presence of the kinase inhibitor to 79.6±4.4% of control when compared to 2'-deoxyadenosine alone, but cell viability was still significantly reduced compared to 5'-amino-5'-deoxyadenosine alone, however.

3.4.9 Effects of saturation of dCK on the anti-cancer activity of adenosine and 2'-deoxyadenosine

Next, to see if the pyrimidine salvage pathway enzyme, dCK, was involved in the anti-cancer activity of adenosine and/or 2-deoxyadenosine, the enzyme was saturated with its natural substrate 2'-deoxycytidine (100 μM; Joachims *et al.* 2008).

In both the MCF-7 and MDAMB231 cells, the presence of 2'-deoxycytidine did not significantly alter the level of inhibition caused by adenosine or 2'-deoxyadenosine (Figure 3.9 A and B) when compared to either compound alone.

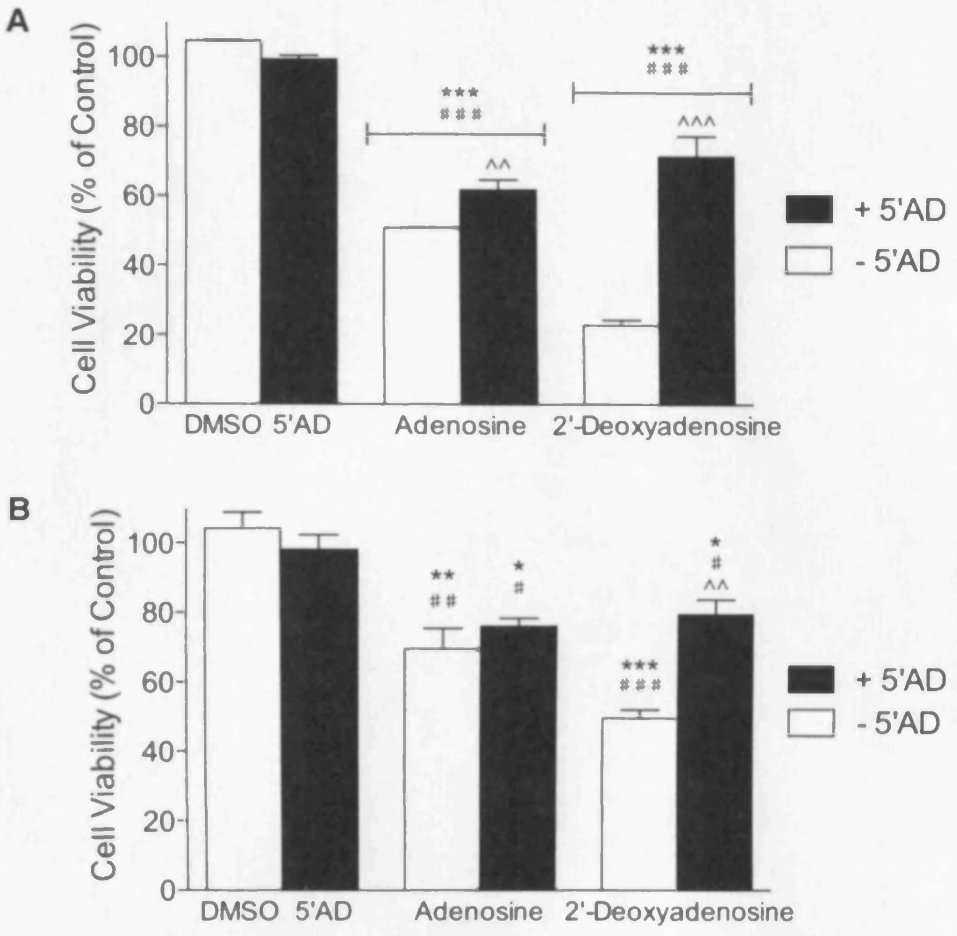


Figure 3.8 Effects of the Adenosine Kinase Inhibitor 5'-Amino-5'-Deoxyadenosine on Adenosine and 2'-Deoxyadenosine Activity on Cell Viability

*The effects of adenosine and 2'-deoxyadenosine (100 μM) on cell viability were investigated in the presence of the adenosine kinase inhibitor, 5'-amino-5'-deoxyadenosine (5'AD; 50 μM) using the MTS assay after 96 hours in MCF-7 (A) and MDAMB231 (B) cells. All adenosine and 2'-deoxyadenosine data are in the presence of deoxycoformycin (1 μM). Data are the mean of three separate experiments repeated in triplicate ± S.E.M, analysed with a one-way ANOVA and Tukey-Kramer post hoc test. *($p<0.05$), **($p<0.01$), ***($p<0.001$) compared to control. #$p(<0.05)$, ##$(p<0.01)$, ###$(p<0.001)$ compared to 5'AD alone. ^^$(p<0.01)$, ^^^$(p<0.001)$ compared to adenosine or 2'-deoxyadenosine alone.*

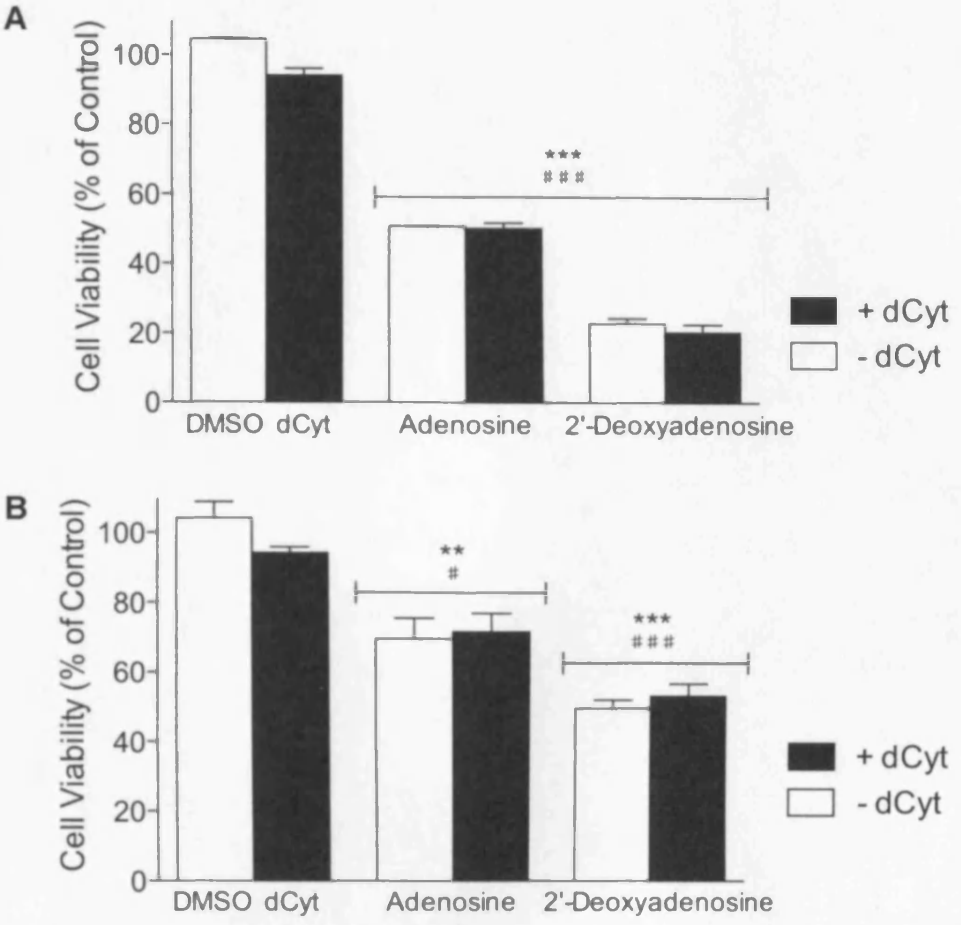


Figure 3.9 Effects of 2'-Deoxycytidine on Adenosine and 2'-Deoxyadenosine Activity on Cell Viability

*The effects of adenosine and 2'-deoxyadenosine (100 μM) on cell viability were investigated in the presence of 2'-deoxycytidine (dCyt; 100 μM) to saturate dCK using the MTS assay after 96 hours in MCF-7 (A) and MDAMB231 (B) cells. All adenosine and 2'-deoxyadenosine data are in the presence of deoxycoformycin (1 μM). Data are the mean of three separate experiments repeated in triplicate ± S.E.M, analysed with a one-way ANOVA and Tukey-Kramer post hoc test. **($p<0.01$), ***($p<0.001$) compared to control. #($p<0.05$), ###($p<0.001$) compared to dCyt alone.*

3.5 Discussion

The aim of this chapter was to determine the expression of adenosine receptors and their function in the regulation of MCF-7 and MDAMB231 cell viability. The effects of manipulating adenosine and 2'-deoxyadenosine levels on the viability of these cells were also investigated.

3.5.1 Adenosine receptors are expressed in both the MCF-7 and MDAMB231 cell lines.

The first aim of this study was to determine the expression of adenosine receptors in the MCF-7 and MDAMB231 cell lines directly at the protein level with Western blotting.

Figure 3.1 very clearly shows that all four adenosine receptors are expressed in both the MCF-7 and MDAMB231 cell lines. Single bands were seen for the A_1, A_{2B} and A_3 receptors which are strongly suggestive of high antibody specificity for the respective proteins. The bands were sized to confirm specificity and all closely matched the expected molecular mass for each receptor. Blots probed for the A_{2A} receptor, however, consistently showed the presence of more than one band. Strong bands appeared at ~48-50 KDa, much stronger than the expected band at 44 KDa, which may suggest that the A_{2A} receptor migrates at a higher MW than expected on SDS-PAGE gels. Piersen *et al.* (1994) confirmed that the A_{2A} receptor has multiple glycosylation sites and, due to this, frequently migrates at 45-50 KDa on SDS-PAGE gels. It is probable that the majority of the A_{2A} receptors are glycosylated, but the receptor also exists in an unglycosylated form.

Previous reports had suggested that the expression of the A_1 receptor could be mediated by ERα in ER-positive MCF-7 cells (Lin *et al.* 2010). However, it appears here that this is not the case as MCF-7 and ER-negative MDAMB231 cells express equal amounts of A_1 receptor. Furthermore, decreased expression of A_1, A_{2A} and A_3 mRNA in rats

following ovariectomy, and thus depletion of oestradiol, suggests the role of ER in regulation of subtypes of adenosine receptors (Rose'Meyer *et al.* 2003). Limited conclusions should be drawn from this study, however, due to difference of species.

The expression of adenosine receptors in breast cancer has been investigated previously, but with contrasting findings. Whilst some studies found that MCF-7 cells did not express A_3 adenosine receptors (Lu *et al.* 2003; Cheung *et al.* 2006), Panjehpour and Karami-Tehrani (2007) found a population of these receptors expressed in this cell line. This study also identified the expression of the other three receptors in MCF-7 and MDAMB468 breast cancer cell lines. Cheung *et al.* (2006) had previously demonstrated a lack of expression of A_1, A_{2B} and A_3 adenosine receptors in T47D, MDAMB231 and SKBR-3 cells, but MCF-7 cells had both A_{2A} and A_{2B} receptors. Experiments by a previous Ph.D student in the Welsh School of Pharmacy (Clark 2004) found that MCF-7 cells (from a different source to the ones used in this thesis) expressed A_1, A_{2B} and A_3 receptors abundantly, as did T47D and MDAMB231 cells. However, none of these cells appeared to express A_{2A} receptors.

These data highlight the differential expression of adenosine receptors in the same cell lines from different groups and therefore it is difficult to compare the results from this thesis with other studies. The variation in expression seen could be due to the different methods used or the variability of the cells from different sources.

3.5.2 Adenosine receptors have a limited role in the proliferation of breast cancer cells

The role of these adenosine receptors in cell proliferation was investigated next. Yao *et al.* (1997) had found that A_3 receptor antagonists induced apoptosis in leukaemia cell lines, and protection from this could be achieved by low concentrations of A_3 receptor

agonists. They suggested that low level adenosine protected the cells via sustained activation of the A_3 receptor. In contrast to this, Synowitz *et al.* (2006) showed that basal A_1 receptor activity by endogenous adenosine retarded glioblastoma cell growth and that removal of this activity resulted in tumour proliferation.

Treatment of both MCF-7 and MDAMB231 cells with selective adenosine receptor antagonists (100 nM; Klotz *et al.* 2000; Kalk *et al.* 2007) had no effect upon cell viability indicating that endogenous activation of adenosine receptors is not required for maintaining cell viability. In section 3.4.6 the non-selective antagonist CGS 15943 also had no effect upon cell viability further confirming these data. These results do not, however, negate the role of adenosine receptors in tumour development *in vivo*. For instance, under states of hypoxia, activation of adenosine receptors is suggested to protect cells from hypoxic damage (Fredholm 2000; Johansson *et al.* 2001). Therefore, adenosine receptor antagonism may be beneficial in tumours *in vivo* where hypoxia occurs. Similarly, adenosine receptor activation in cells of the immune system that are normally involved in destroying cancer cells such as natural killer and other T cells, can inhibit their anti-cancer function (Raskovalova *et al.* 2006).

The effects of adenosine receptor activation on cell viability were then investigated. Numerous reports have shown that adenosine receptor activation by the endogenous ligand adenosine, and selective and non-selective agonists causes apoptosis or reduced proliferation in a number of different cancer cells (Tey *et al.* 1992; Kohno *et al.* 1996; Colquhoun and Newsholme 1997; Yao *et al.* 1997; Fishman *et al.* 2000; Ohana *et al.* 2001; Merighi *et al.* 2002; Lee *et al.* 2005; Sai *et al.* 2006; Panjehpour and Karami-Tehrani 2007; Bar-Yehuda *et al.* 2008; Kim *et al.* 2008).

In the present study, the non-selective agonist, NECA was used to activate adenosine receptors and cell viability was then measured.

There was no effect on cell viability in the MDAMB231 cells with NECA at concentrations up to 100 μM indicating that adenosine receptor activation does not influence cell proliferation or death. However, at concentrations of 30 and 100 μM, NECA significantly reduced cell viability in the MCF-7 cells by 20 and 30%, respectively. As NECA has *Ki* values in the low nanomolar range for A_1, A_{2A} and A_3, and in the low micromolar range for the A_{2B} receptor (Klotz *et al.* 2000) these effects are likely to be non-selective effects. This could be tested with an adenosine receptor antagonist to see if the effects of NECA could be blocked. However, this is unlikely to have much clinical potential as such high concentrations of NECA would probably affect adenosine signalling elsewhere and so was not investigated further.

3.5.3 Endogenous adenosine and/or 2'-deoxyadenosine are essential for normal cell function.

It was then decided to focus the studies on the effects of the endogenous molecules, adenosine and 2'-deoxyadenosine.

The effects of eliminating adenosine and 2'-deoxyadenosine from the cells and surrounding matrix were investigated using ADA, the enzyme responsible for metabolism of adenosine and 2'-deoxyadenosine by deamination into inosine and 2'-deoxyinosine, respectively (Bañales *et al.* 1999). Adenosine deaminase is found mostly as a cytostolic enzyme, but is also attached to the outer cell membrane anchored by the CD26 membrane protein (Romanowska *et al.* 2007).

The addition of adenosine deaminase (2U/mL; Lopes *et al.* 1999) produced a highly significant reduction in viable cells compared to control in both MCF-7 and MDAMB231 cell lines by 60 and 80%, respectively. This inhibition was significantly greater in the MDAMB231 cells compared to the MCF-7 cells, suggesting that removal of adenosine/2'-deoxyadenosine is significantly more detrimental to cell function in these cells. 2'-Deoxyadenosine is a better substrate for ADA

than adenosine (K_m 34 and 48 μM, respectively; Wiginton *et al.* 1981) and is therefore likely that the addition of ADA to the extracellular environment is depleting deoxynucleotides more readily than adenosine levels.

These data are consistent with the role of adenosine and 2'-deoxyadenosine as precursors to vital molecules such as ATP and cAMP, and for DNA synthesis and repair. The difference between the two cell lines could possibly be explained by the MDAMB231 cells being more metabolically active and thus requiring a higher turnover of ATP and increased DNA synthesis. Turturro *et al.* (2004) found that MDAMB231 cells have increased basal lactate production compared to MCF-7 cells, indicating an increase in glycolysis and thus metabolic activity which may explain these data.

An alternative explanation for these data is that the effects seen are not due to depletion of adenosine or 2'-deoxyadenosine, but rather a build up of the metabolites inosine or 2'-deoxyinosine, respectively. This hypothesis was tested by the addition of a high concentration (100 μM) of inosine to the cells. As inosine had no effect on cell viability in either cell line, the reduction in cell viability is likely to be a result of adenosine/2'-deoxyadenosine depletion.

3.5.4 Increasing endogenous adenosine and 2'-deoxyadenosine levels does not affect cell viability

Deoxycoformycin is a clinically used drug in the treatment of non-Hodgkin's lymphoma, B-cell chronic lymphocytic leukaemia and Waldenström's macroglobulinemia (Ho and Hensel 2006). Deoxycoformycin is an irreversible inhibitor of ADA and was used in this study to investigate what effects increasing the endogenous concentration of adenosine and 2'-deoxyadenosine would have on cell viability.

Deoxycoformycin at a concentration of 1 μM (Aye *et al.* 1982) had no effect upon cell viability in either MCF-7 or MDAMB231 cells. This indicates that accumulation of adenosine or/and 2'-deoxyadenosine is not sufficient to affect the viability of these cell lines. It would have been interesting to be able to quantify the accumulation of adenosine and 2'-deoxyadenosine in these cells. A HPLC method was tested but the results proved inconsistent and it was not pursued.

These data show that increasing endogenous adenosine and 2'-deoxyadenosine levels do not affect cell viability. Ogawa *et al.* (2000) found similar results in isolated hairy cell leukaemia cells with deoxycoformycin, but in the presence of 10 μM 2'-deoxyadenosine, cell viability was reduced by almost 10-fold compared to 2'-deoxyadenosine alone. This point suggests that deoxycoformycin should be investigated in the presence of exogenous adenosine or 2'-deoxyadenosine.

3.5.5 Exogenous adenosine and 2'-deoxyadenosine significantly reduces cell viability

There have been numerous studies suggesting that increasing exogenous levels of adenosine and 2'-deoxyadenosine can lead to an inhibition of cell proliferation and result in cell death. This has been demonstrated in a number of cell lines to occur by an intracellular mechanism (Tanaka *et al.* 1994; Barry and Lind 2000; Saitoh *et al.* 2004; Schneider *et al.* 2001; Hashemi *et al.* 2005; Wu *et al.* 2006). A number of studies have also identified an extracellular mechanism of action in cell lines (Kohno *et al.* 1996; Colquhoun and Newsholme 1997; Yao *et al.* 1997; Fishman *et al.* 2000; Ohana *et al.* 2001; Merighi *et al.* 2003; Panjehpour and Karami-Tehrani 2004; Lee *et al.* 2005; Sai *et al.* 2006; Panjehpour and Karami-Tehrani 2007; Bar-Yehuda *et al.* 2008; Kim *et al.* 2008).

In preliminary studies, adenosine and 2'-deoxyadenosine alone had no effect on cell viability, probably due to their metabolism by ADA. This

has been demonstrated previously in other breast cancer cell lines (Hashemi *et al.* 2005) and Weisman *et al.* (1988) had also found that 125 μM adenosine was completely metabolised to inosine when added to fibroblasts in 8 hours. Therefore, for all studies involving adenosine and 2'-deoxyadenosine, 1 μM deoxycoformycin was used to inhibit the activity of ADA. The metabolism of adenosine and 2'-deoxyadenosine by intracellular phosphorylation was not inhibited because this is one of the proposed mechanisms of action of adenosine/2'-deoxyadenosine toxicity (Hashemi *et al.* 2005).

Adenosine was able to reduce the number of viable MCF-7 and MDAMB231 cells, but at a 10-fold lower concentration in the MDAMB231 cells (10 μM). There were no significant differences between the IC_{50} values of adenosine, however. Similarly, there was no difference in the activity of 2'-deoxyadenosine on cell viability in either cell line nor when compared to the activity of adenosine. In both the MCF-7 and MDAMB231 cells, concentrations of 2'-deoxyadenosine ≥30 μM significantly reduced cell viability. Hashemi *et al.* (2005) also found that adenosine and 2'-deoxyadenosine inhibited the growth of MCF-7 and MDAMB436 (ER-negative) breast cancer cells with equal potency but at concentrations ≥250 μM.

The extracellular concentration of adenosine in most tissues is between 10-200 nM under normal physiological function (Daines *et al.* 2003). Under states of hypoxia, extracellular adenosine levels can increase up to 40 μM (Schubert *et al.* 1994). In a mouse model of solid tumours, extracellular adenosine reached 2.5 μM, and in the presence of an ADA and adenosine kinase inhibitor, reached 13 μM (Blay *et al.* 1997). On this basis, neither adenosine nor 2'-deoxyadenosine are likely to be effective anti-cancer drugs as the concentrations that could be maintained in the extracellular fluid are unlikely to be sufficient to reduce cell viability.

The role of adenosine in tumour function should not be discarded, however, as it is known to influence tumour fate by stimulating angiogenesis, increasing tumour motility and is an immunosuppressant at hypoxic concentrations (Blay *et al.*1997; Lindon 2006; Merighi *et al.* 2007; Ryzhov *et al.* 2007). Also, determining the mechanism of action of adenosine and 2'-deoxyadenosine may provide insights into novel pathways that can be targeted for future use. It may also provide an insight into how the clinically used adenosine and 2'-deoxyadenosine analogues, cladribine, clofarabine and fludarabine, may work in these cells.

Interestingly there appeared to be a biphasic response to adenosine in both cell lines that did not occur with 2'-deoxyadenosine. The actions of adenosine in the MDAMB231 cells appear to be saturated at 30 μM and at 100 μM in the MCF-7 cells. This effect could be explained in two ways, either different mechanisms of action between adenosine and 2'-deoxyadenosine or, adenosine requires conversion to 2'-deoxyadenosine and this step is rate-limiting. The mechanisms of action of adenosine and 2'-deoxyadenosine are discussed in the next section.

3.5.6 Effects of adenosine are part-mediated by adenosine receptor activation in the MCF-7 cells

There is a disagreement in the literature regarding the role of adenosine receptor activation, with most studies suggesting a role in initiating apoptosis (Kohno *et al.* 1996; Colquhoun and Newsholme 1997; Yao *et al.* 1997; Fishman *et al.* 2000; Ohana *et al.* 2001; Merighi *et al.* 2003; Panjehpour and Karami-Tehrani 2004; Lee *et al.* 2005; Sai *et al.* 2006; Panjehpour and Karami-Tehrani 2007; Bar-Yehuda *et al.* 2008; Kim *et al.* 2008). In contrast Hashemi *et al.* (2005) previously demonstrated that the cytotoxicity caused by adenosine and 2'-deoxyadenosine in MCF-7 cells was independent of adenosine receptor activation. However, studies by Panjehpour and Karami-Tehrani (2004 and 2007)

demonstrated that A_3 adenosine receptor activation by selective agonists and adenosine caused apoptosis in MCF-7 and MDAMB468 cells.

In both the MCF-7 and MDAMB231 cells, pre-treatment with the non-selective adenosine receptor antagonist, CGS 15943, had no effect upon 2'-deoxyadenosine activity on cell viability. This was expected as 2'-deoxyadenosine is not an agonist for adenosine receptors (Schrier *et al.* 2001). Similarly, CGS 15943 had no effect upon the action of adenosine in the MDAMB231 cells, indicating a non-receptor mediated mechanism of action for adenosine in these cells. However, CGS 15943 did significantly attenuate the effects of adenosine in the MCF-7 cells, but not completely, indicating that the effects of adenosine on cell viability are mediated in part by adenosine receptor activation, but adenosine is also working by another mechanism. The use of selective antagonists could help elucidate which receptor(s) adenosine is activating to produce this response. Although most evidence suggests the A_3 receptor is primarily responsible for initiating apoptosis or decreasing cell viability (Kohno *et al.* 1996; Fishman *et al.* 2000; Ohana *et al.* 2001; Panjehpour and Karami-Tehrani 2004; Lee *et al.* 2005; Panjehpour and Karami-Tehrani 2007; Bar-Yehuda *et al.* 2008; Kim *et al.* 2008), there is evidence to show the involvement of A_1 (Tey *et al.* 1992; Colquhoun and Newsholme 1997; Sai *et al.* 2006) and A_{2A} receptors (Merighi *et al.* 2002) in these processes.

To further this work, the effects of adenosine and 2'-deoxyadenosine on adenosine receptors were investigated by looking at their effects on ERK1/2 phosphorylation. Adenosine receptors are known to be positively coupled to the ERK 1/2 pathway in naïve and adenosine receptor transfected cell lines (Feoktistov *et al.* 1999; Seidel *et al.* 1999; Fredholm *et al.* 2000; Robinson and Dickenson 2001; Zhang and Liu 2002; Hammarberg *et al.* 2003; Schulte and Fredholm 2003; Rivo *et al.* 2007). The data clearly show that the MDAMB231 cells have increased

basal ERK 1/2 signalling compared to MCF-7 cells, and this has been shown previously (Chen *et al.* 2009).

In the MCF-7 cells, adenosine was able to increase ERK 1/2 phosphorylation compared to control, and in the presence of CGS 15943, this effect was reduced. In contrast, 2'-deoxyadenosine did not influence the phosphorylation of ERK 1/2, consistent with the fact that it is not a ligand for adenosine receptors (Schrier *et al.* 2001). This strongly suggests that adenosine activates adenosine receptors in MCF-7 cells and is consistent with the cell viability data. It is interesting, however, that adenosine receptor activation–induced ERK 1/2 phosphorylation is correlated with a decrease in cell viability considering the role of ERK 1/2 is generally associated with mitogenic signalling (Schulte and Fredholm 2003; Fang and Richardson 2005). However, numerous studies have also implicated transient ERK 1/2 activation in the initiation stages of apoptosis (Mohr *et al.* 1998; Blagosklonny 1999; Ishikawa and Kitamura 1999).

When the effects of adenosine and 2'-deoxyadenosine were investigated in the MDAMB231 cells, both adenosine and 2'-deoxyadenosine greatly reduced ERK 1/2 phosphorylation compared to control. As CGS 15943 did not antagonise these effects it can be assumed that these effects are adenosine receptor-independent. Whilst it has been shown previously that adenosine receptor activation by adenosine and various receptor agonists can decrease ERK 1/2 signalling (Dubey *et al.* 2005; Jijon *et al.* 2005; Tan *et al.* 2006; Merighi *et al.* 2007; Bieber *et al.* 2008), this does not appear to be the case here.

Adenosine monophosphate-activated protein kinase (AMPK) controls cellular energy metabolism. Activation of AMPK encourages the cell to produce ATP for energy whilst reducing ATP-consuming processes, including cell proliferation and division (Towler and Hardie 2007). Adenosine has been demonstrated to activate AMPK in gastric cancer

and hepatoma cell lines (Saitoh *et al.* 2004; Aymerich *et al.* 2006) and Baumann *et al.* (2007) found that activation of AMPK inhibited the phosphorylation of ERK. The exact role of AMPK in the inhibition of ERK signalling is still unclear, but it may explain the findings in the MDAMB231 cells described here.

3.5.7 The effects of 2'-deoxyadenosine on cell viability are mediated by intracellular phosphorylation by adenosine kinase

As the effects of 2'-deoxyadenosine, and some of the effects of adenosine on cell viability were not mediated by adenosine receptor activation, the role of intracellular phosphorylation was investigated.

Several studies have established that intracellular phosphorylation of adenosine or 2'-deoxyadenosine by adenosine kinase is responsible for the anti-proliferative mechanisms of action in a variety of cancer and normal tissue cell lines. Toxicity is suggested to arise from the accumulation of the nucleotide triphosphate that causes an imbalance of intracellular nucleotides, inhibition of ribonucleotide reductase, inhibition of *S*-adenosylhomocysteine hydrolase and DNA base pair mismatches. These effects ultimately lead to impaired repair and synthesis of DNA leading to apoptosis or cellular quiescence (Tanaka *et al.* 1994; Wakade *et al.* 1995; Kulkarni *et al.* 1998; Barry and Lind 2000; Schreir *et al.* 2001; van der Weile *et al.* 2002; Saitoh *et al.* 2004; Hashemi *et al.* 2005). Furthermore, the anti-cancer actions of the clinically used analogues cladribine, clofarabine and fludarabine, which are 2'-deoxyadenosine analogues, are known to be due to phosphorylation to their triphosphate derivatives (Månsson *et al.* 1999; Bonate *et al.* 2006).

The adenosine kinase inhibitor 5'-amino-5'-deoxyadenosine (50 μM Barry and Lind 2000) was used to inhibit the actions of this enzyme. Alone, 5'-amino-5'-deoxyadenosine had no significant effect on either cell line indicating that the cells can probably switch from the salvage

pathway to the *de novo* pathway (see Figure 1.5, Chapter 1.4.1) in order to produce purine nucleotides (Hatse *et al.* 1999).

In both cell lines, in the presence of 5'-amino-5'-deoxyadenosine, the anti-cancer activity of 2'-deoxyadenosine was significantly attenuated, implying strongly that 2'-deoxyadenosine requires the action of adenosine kinase to impart its anti-cancer activity. This is most likely a direct effect of phosphorylation on 2'-deoxyadenosine by adenosine kinase to dAMP. Camici *et al.* (1995) and Giannecchini *et al.* (2003) both identified phosphorylation by adenosine kinase as an essential step in the toxicity of 2'-deoxyadenosine in colon cancer cells, as did Hashemi *et al.* (2005) in breast cancer cells. Interestingly, the effects of adenosine were significantly attenuated, albeit less so than with 2'-deoxyadenosine, in the presence of 5'-amino-5'-deoxyadenosine in the MCF-7 cells, but not in the MDAMB231 cells. This implies that adenosine also requires phosphorylation by adenosine kinase in MCF-7 cells which has been reported previously (Tanaka *et al.* 1994; Wakade *et al.* 1995; Kulkarni *et al.* 1998; Barry and Lind 2000; Schreir *et al.* 2001; van der Weile *et al.* 2002; Saitoh *et al.* 2004). As the anti-cancer activity of 2'-deoxyadenosine in the MDAMB231 and MCF-7 cells, and adenosine in the MCF-7 cells was not completely attenuated by 5'-amino-5'-deoxyadenosine, it may indicate another mechanism of action behind adenosine and 2'-deoxyadenosine toxicity. Alternatively, it is possible that other routes of phosphorylation are also contributing as the concentration of 5'-amino-5'-deoxyadenosine used here should be sufficient to inhibit completely the activity of adenosine kinase (Sinclair *et al.* 2001).

The reasons for the differences observed here between adenosine and 2'-deoxyadenosine in combination with the adenosine kinase inhibitor are unclear. Giannecchini *et al.* (2003) demonstrated that 5'-amino-5'-deoxyadenosine inhibited the formation of dATP, but not ATP and confirmed that it is indeed dATP and not ATP accumulation that is toxic

to cells. Whilst 2'-deoxyadenosine must clearly go via a route of adenosine kinase phosphorylation to dATP, it appears that adenosine does not. It is possible that adenosine is phosphorylated by another kinase to AMP. As the purine analogues cladribine and clofarabine are phosphorylated by dCK to their monophosphates, it is possible that dCK is also phosphorylating adenosine here. However, the addition of 2'-deoxycytidine sufficient to competitively inhibit the action of dCK (100 μM; Joachims *et al.* 2008) had no effect on adenosine or 2'-deoxyadenosine toxicity in either cell line. This implies that dCK does not phosphorylate adenosine or 2'-deoxyadenosine.

The anti-cancer action of adenosine in the MCF-7 cells may be mediated by both adenosine receptor activation and intracellular phosphorylation. However, the action of adenosine in the MDAMB231 cells appears to be mediated by neither of these mechanisms. There have been other proposed mechanisms of action of adenosine in mediating apoptosis including inhibition of methyltransferases (Rounds *et al.* 1998; Hermes *et al.* 2008) or direct initiation of caspase 3 activity (Wu *et al.* 2006). The latter may also explain the differences seen between the MCF-7 and MDAMB231 cells as MCF-7 cells are caspase 3-deficient but MDAMB231 cells are not (Yang *et al.* 2007) so adenosine may be mediating its effects via caspase 3 activation in the MDAMB231 cells. Further investigation of the mechanisms of action of adenosine and 2'-deoxyadenosine is needed.

3.6 Conclusion

Both the MCF-7 and MDAMB231 cell lines express A_1, A_{2A}, A_{2B} and A_3 adenosine receptors at the protein level but the data indicate the limited role of direct adenosine receptor ligands as a treatment for breast cancer.

Whilst endogenous adenosine and 2'-deoxyadenosine were demonstrated to be essential for normal cell function, exogenous administration, although decreasing cell viability in both cell lines, would have little direct clinical potential against breast cancer.

In deciphering the mechanisms behind adenosine and 2'-deoxyadenosine toxicity, activation of adenosine receptors was important for adenosine activity in the MCF-7 cells, as was intracellular phosphorylation by adenosine kinase, suggesting a dual mechanisms of action of adenosine in these cells. The action of 2'-deoxyadenosine appeared to be mediated mostly by intracellular phosphorylation.

In the MDAMB231 cells, phosphorylation of 2'-deoxyadenosine by adenosine kinase was also essential for its function, but this was not the case with adenosine. However, both adenosine and 2'-deoxyadenosine reduced ERK 1/2 phosphorylation independently of adenosine receptor activation suggesting a novel mechanism of action of these compounds in this cell line. This may be linked to the phosphorylation mechanism of 2'-deoxyadenosine but a definitive answer remains to be elucidated.

These data show that intracellular phosphorylation of 2'-deoxyadenosine is effective at reducing cell viability. Whilst 2'-deoxyadenosine is unlikely to have much benefit clinically, the 2'-deoxyadenosine analogues cladribine and clofarabine are very effective against haematological malignancies clinically and also require internal phosphorylation for their activity. Therefore, the effects of these analogues were investigated in the next chapter.

Chapter 4: Pharmacological Investigation of the Activity of PNAs in Breast Cancer Cells

4.1 Introduction

PNAs represent a significant number of anti-cancer drugs used clinically and adenine PNAs include deoxycoformycin (ADA inhibitor), cladribine, fludarabine and clofarabine (halogenated analogues).

Halogenated PNAs require active transport across the plasma membrane via nucleoside transporters (NTs) (King *et al.* 2006) and are then phosphorylated primarily by dCK to the monophosphate. They are then sequentially phosphorylated by a purine monophosphate kinase and a nucleoside purine diphosphate kinase to the biologically active di- and triphosphate forms, respectively (Lotfi *et al.* 1999; Månsson *et al.* 1999; Galmarini *et al.* 2001).

The triphosphate forms of fludarabine, cladribine and clofarabine are all potent inhibitors of ribonucleotide reductase (Griffig *et al.* 1989; Bontemps *et al.* 2000; Galmarini *et al.* 2001; Cerqueira *et al.* 2005; Bonate *et al.* 2006) which results in a decrease in available dNTPs for DNA synthesis and repair and results in cell cycle arrest or apoptosis. These PNA can also be inserted into the DNA template leading to DNA strand breakage and inhibition of DNA polymerases which can initiate apoptosis (Huang *et al.* 1990; Huang and Plunkett 1991; Bontemps *et al.* 2000; Genini *et al.* 2000a; Bonate *et al.* 2006).

Several other mechanisms have also been attributed to the cytotoxic effects of these drugs including disruption of mitochondrial membrane potential (Marzo *et al.* 2001), inhibition of DNA ligase and DNA primase (Catapano *et al.* 1991; Yang *et al.* 1992), activation of PARP and subsequent depletion of NAD^+ and ATP (Pettitt *et al.* 2000) and direct interaction and release of various mitochondrial apoptosis-inducing molecules including cytochrome C, AIF, caspase 9 and APAF 1 (Xie and Plunkett 1996).

Clinically, these drugs are used in the treatment of various haematological malignancies including hairy cell leukaemia, chronic lymphocytic leukaemia, Waldenström's macroglobulinaemia, acute leukaemia and non-Hodgkin's lymphoma (Bryson *et al.* 1993; Fidias *et al.* 1996; Lenz *et al.* 2004; Faderl *et al.* 2005; Ho and Hensel 2006; Robak and Robak 2007). Their action against solid tumours is less well documented. However, gemcitabine, which is a pyrimidine NA and has similar mechanisms of action to the PNA, is active against a wide variety of solid tumours (Kroep *et al.* 2002). Few *in vivo* studies of clofarabine in solid tumours have been undertaken but Kantarjian *et al.* (2003) identified that in 13 patients with solid tumours treated with clofarabine, including breast (n=7), no specific response to clofarabine was achieved. However, they did note that one metastatic breast cancer patient (undefined breast cancer) did regain stable disease levels for 9 months on clofarabine treatment, suggesting some benefit. Cunningham *et al.* (2005), in a study of 57 patients with solid tumours (pancreatic, lung, colorectal, prostate, bladder and laryngeal, but not breast cancer) stated that clofarabine was well tolerated by patients, and was showing good clinical activity against a number of different tumours.

Several studies have looked at the effects of clofarabine *in vitro* in a variety of solid tumour cell lines including colon, pancreatic, renal, lung and melanoma cell lines and also in a number of mice models *in vivo*. Their studies identified good activity with IC_{50} values in the high nM range and curative to moderate activity in mouse solid tumour xenograft models (Takahashi *et al.* 1999; Waud *et al.* 2000). Hashemi *et al.* (2003; 2004) investigated cladribine activity in MDA-MB-468 and MCF-7 cells breast cancer cells, respectively. The studies identified cladribine as being able to reduce cell viability and induce apoptosis in both cell lines, however, at relatively high μM concentrations.

In the previous chapter, 2'-deoxyadenosine was found to significantly reduce cellular viability and this was attributed to intracellular phosphorylation by adenosine kinase. Analogues of 2'-deoxyadenosine already in the clinic are desirable for therapeutic use based upon their high tolerability profiles compared to standard cytotoxics. There is therefore a need to investigate these drugs further in solid tumours, including breast cancer, to evaluate their potential for such use.

4.2 Chapter Aims

The aims of this chapter were:

- To determine the pharmacological activity of clinically used NAs against a variety of human breast cancer cell lines, and the control leukaemia cells, HL60.
- To investigate the mechanism(s) of action of these drugs using pharmacological methods.

4.3 Methods

The protocols for the MTS, cell growth and annexin V apoptosis assay are described in detail in Chapter 2.

4.3.1 MTS Assay

Cells were seeded at 5000 cells/well into 96 well plates and allowed to plate down overnight. Media was aspirated from the adherent cells and replaced with drug-containing media. Concentration-response curves were produced for clofarabine, cladribine, fludarabine and gemcitabine at concentrations between 0.1 nM and 100 μM. For the 2'-deoxycytidine (100 μM) and hydroxyurea (300 μM) assays, 1 μM of clofarabine and cladribine and 10 μM of fludarabine were used. Relevant media only, vehicle (DMSO; highest concentration was 0.5%) and blank media (no cells) controls were included on each plate. Cells were then left for 96 hours (apart from the time course assay) at 37°C in 5% CO_2 in a humidified atmosphere. The MTS assay was then performed as described earlier (section 2.3.2).

4.3.2 Annexin V FACS Apoptosis Assay

Cells were seeded into 25 cm^2 cell culture flasks and allowed to plate down overnight. The media was then replaced with media only, vehicle (DMSO: 0.005%) or 1 μM clofarabine and cells were left for 96 hours at 37°C in 5% CO_2 in a humidified atmosphere. After this time, detached cells were collected by centrifugation, and cells still attached were detached using trypsin/EDTA. All cells were collated together, washed in PBS twice and then resuspended in annexin V binding buffer and passed through a 21G needle. Annexin V and PI were then added and the cells were immediately analysed using the FL1 and FL2 channels on the flow cytometer as described (section 2.4.3).

The data are expressed as a % of cells positively stained for annexin V or/and PI, corrected for the vehicle (clofarabine treated – DMSO

vehicle). This accounts for any effect the vehicle is having, and also any false positives caused by processing the samples.

4.3.3 Cell Growth Assay

Cells were seeded into 24 well plates and allowed to settle overnight. The media was then changed the next day and cells allowed to grow for the required amount of time, 24, 48 and 96 hours. At each time point, cells were harvested with trypsin/EDTA, suspended as a single cell suspension and counted in a Beckman Coulter Counter.

4.3.4 Data Analysis

All data are expressed as mean ± S.E.M. except for the IC_{50} data which are expressed as the mean and the 95% C.I. Data were entered into GraphPad Prism V5.0b and graphs were plotted. IC_{50} values are the 50% inhibition between the top and bottom of the curve. They were calculated using non-linear curve regression with a fixed Hill slope according to the following formula:

$$y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(X - \text{Log } IC_{50})}}$$

Where:

y = response

x = log (drug concentration)

Top = y value at the top of the plateau

Bottom = y value at the bottom of the plateau

Comparisons between two groups were made using a Student's *t* test and a one-way ANOVA was used for three or more groups with a Tukey-Kramer *post hoc* test if a significant difference was observed or a Bonferroni *post hoc* test when comparing only selected pairs of data. Probability values of less than 0.05 were considered statistically significant.

4.4 Results

4.4.1 Molecular profile of breast cancer cell lines

A literature search was carried out firstly to identify the molecular profile of the different breast cancer cell lines used for this study. The data for this are expressed in Table 4.1 and are adapted from Liang *et al.* (2005) and Neve *et al.* (2006). Three ER-positive and three ER-negative cell lines, two of which were triple negative breast cancer (TNBC) cell lines (MDAMB231 and MDAMB436), were used. Two Her2 overexpressing cell lines are also included, BT 474 and SKBR-3.

4.4.2 Activity of clofarabine in breast cancer cell lines

Concentration-response curves show the effects of clofarabine on cell viability against the different cell lines (Figure 4.1 A). The IC_{50} values for clofarabine in the ER-positive breast cancer cell lines were significantly higher compared to the HL60 cells ($p<0.01$) and ER-negative cells ($p<0.05$) by 15 and 8-fold, respectively (Figure 4.1 B). There were no differences between the IC_{50} values or % efficacy of clofarabine at 1 μM in the ER-negative cell lines compared to the HL60 cells. The % efficacy of clofarabine at 1 μM was significantly reduced ($p<0.05$) in the MCF-7 cells compared to the HL60, MDAMB231 and MDAMB436 cell lines. It was also significantly reduced ($p<0.05$) in the BT 474 cells compared to the HL60 and MDAMB436 cells (Figure 4.1 B). There was no significant difference in the % efficacy at 1 μM of clofarabine between the T47D cells and ER-negative and HL60 cells.

4.4.3 Activity of cladribine and fludarabine in MCF-7, MDAMB231 and HL60 cell lines

The activity of the older generation PNAs, cladribine and fludarabine, were then investigated. The effects were only studied in the ER-positive and ER-negative cell lines, MCF-7 and MDAMB231, respectively, as well as the HL60 cell line.

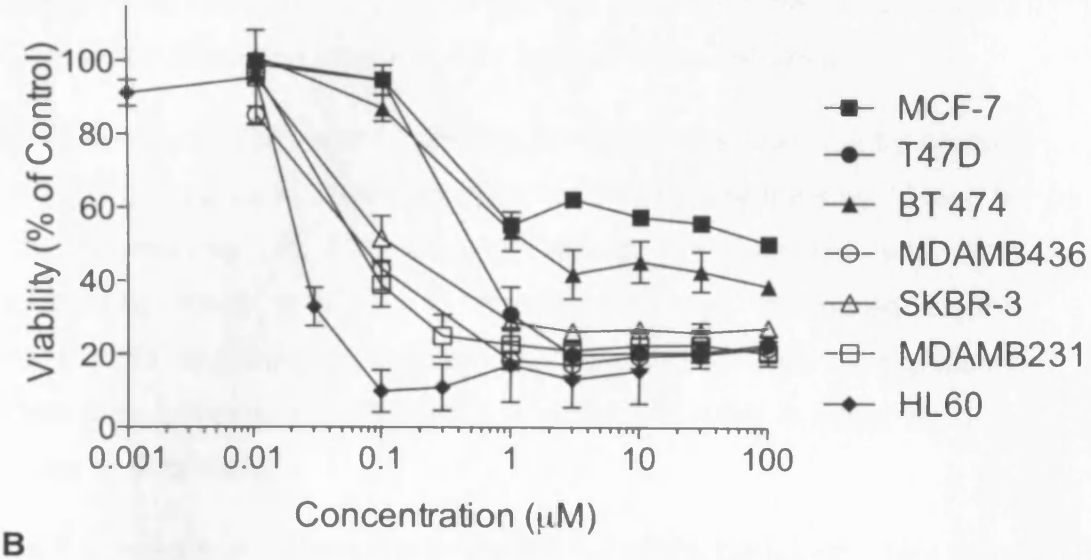
Cell Line	ER	PR	Her2	p53	Class	Type
MCF-7	+	+	-	+	Lu	IDC
T47D	+	+	-	$+^{M}$	Lu	IDC
BT 474	+	+	+	$+^{M}$	Lu	IDC
MDAMB231	-	-	-	$+^{M}$	BaB	AC
MDAMB436	-	-	-	-	BaB	IDC
SKBR-3	-	-	+	+	Lu	AC

Table 4.1 Molecular Profile of Breast Cancer Cell Lines.

The table describes the molecular profiles of the six different breast cancer cell lines used in this chapter with data taken from the literature rather than experimental work undertaken during this thesis. Data collated from (Liang et al. 2005; Neve et al. 2006).

ER – Oestrogen Receptor
PR – Progesterone Receptor
Her2 – Epidermal Growth Factor Receptor 2
p53 – Tumour Suppressor Protein, p53
Class – Gene cluster profile. Lu = Luminal; BaB = Basal Type B
Type – Cancer Type; IDC = invasive ductal carcinoma; AC = adenocarcinoma
+ indicates the presence of the protein; - absence of the protein; $+^{M}$ is protein present but dysfunctional/mutated

A



B

Cell Line	IC_{50} values (nM)	% Efficacy at 1 μM	IC_{50} value change
MCF-7	336 (252 – 419)**#	44.6 (0.8)*1	15.6
T47D	315 (100 – 530)**#	69.0 (7.4)	15.8
BT 474	308 (-69 – 685)**#	46.4 (5.4)*2	15.4
MDAMB231	37 (-11 – 85)	77.2 (4.6)	2.0
MDAMB436	45 (-32 – 121)	81.6 (2.4)	2.2
SKBR-3	45 (31 – 120)	71.1 (1.4)	2.2
HL60	20 (-14 – 54)	82.9 (10.1)	-

Figure 4.1 Effect of Clofarabine on Breast Cancer Cell Viability

*A shows the concentration-response curve of the cell lines to clofarabine after 96 hours treatment. The table (B) gives the IC_{50} values (95% C.I.), the % response of control to clofarabine at 1 μM (S.E.M) and the fold change in IC_{50} values compared to HL60 cells. Data are expressed as the mean of at least three independent experiments, repeated in triplicate. *($p<0.05$), **($p<0.01$) compared to HL60 cells; #($p<0.05$) compared to ER-negative cell lines. [1]($p<0.05$) compared to MDAMB231 and MDAMB436 cells; [2]($p<0.05$) compared to MDAMB436 cells. Data were analysed with one-way ANOVA and the Tukey-Kramer post hoc test. Closed symbols (ER-positive cells); open symbols (ER-negative cells).*

Concentration-response curves show the activity of cladribine (Figure 4.2 A) and fludarabine (figure 4.3 A) against these cell lines.

The IC_{50} value of cladribine in the MCF-7 cells was significantly greater ($p<0.01$) compared to the MDAMB231 and HL60 cell lines by 11 and 9-fold, respectively. At 0.1 μM, the efficacy of cladribine was also significantly lower ($p<0.05$) in the MCF-7 cells compared to the MDAMB231 and HL60 cells (Figure 4.2 B). There were no significant differences between the MDAMB231 and HL60 cells in either the IC_{50} values or % efficacy.

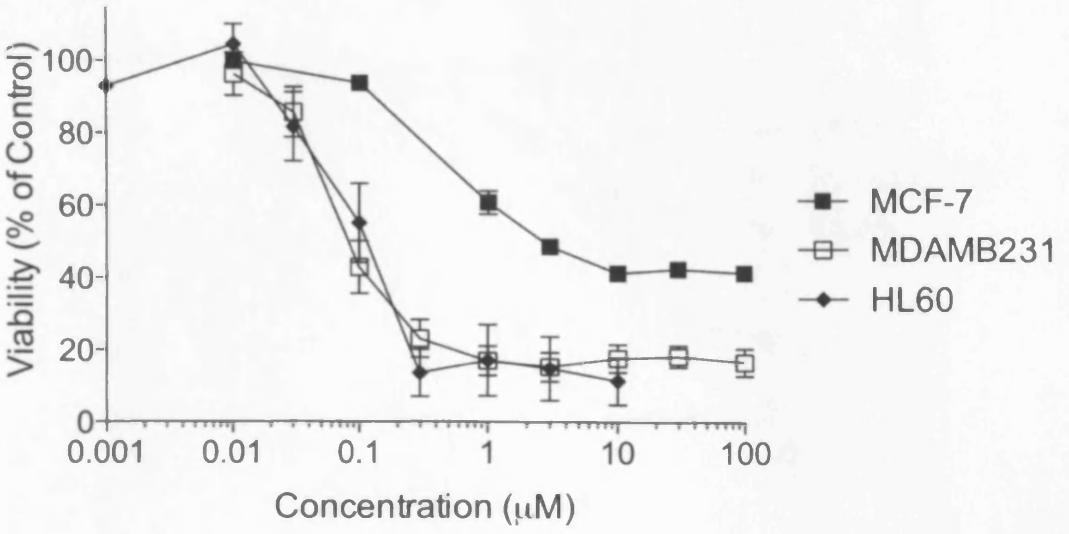
The IC_{50} values of fludarabine in the MCF-7 and MDAMB231 cells were significantly greater ($p<0.001$ and $p<0.01$, respectively) compared to the HL60 cells by 103 and 57-fold, respectively. The IC_{50} value of fludarabine in the MDAMB231 cells was significantly lower ($p<0.05$) compared to the MCF-7 cells. The efficacy of fludarabine at 10 μM was significantly greater ($p<0.001$) in the HL60 cells compared to both the MCF-7 and MDAMB231 cell lines (Figure 4.3 B).

4.4.4 Activity of gemcitabine in MCF-7 and MDAMB231 cells

The activity of the pyrimidine NA, gemcitabine, was then investigated in the MCF-7 and MDAMB231 cell lines. Concentration-response curves show the effects of gemcitabine in these cell lines (Figure 4.4 A).

The IC_{50} values of gemcitabine and efficacy at 1 μM are shown in Figure 4.4 B. The efficacy of gemcitabine was significantly smaller ($p<0.01$) at 1 μM in the MCF-7 compared to the MDAMB231 cells but there were no significant differences between the IC_{50} values.

A



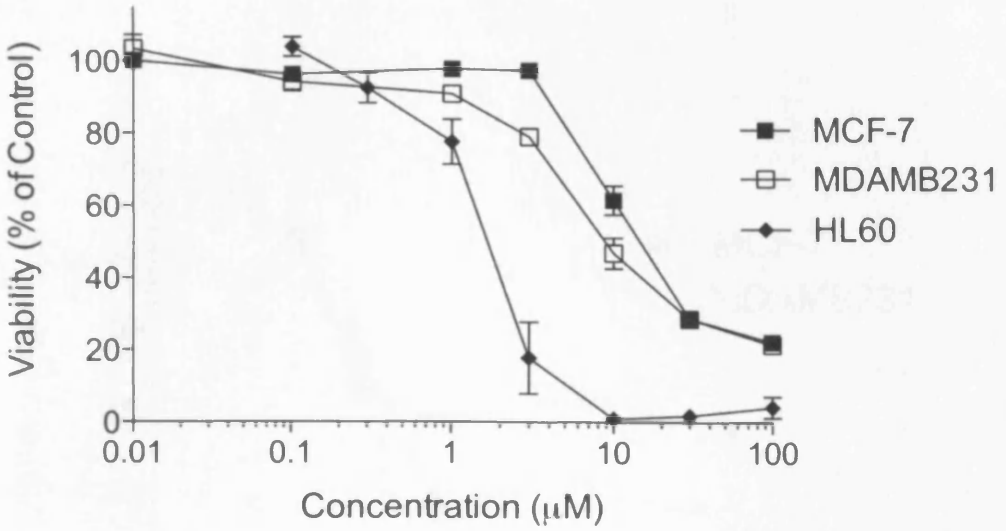
B

Cell Line	IC_{50} value (nM)	% Efficacy at 0.1 μM	IC_{50} value change
MCF-7	513 (58 – 968)**	6.1 (1.8)*	9
MDAMB231	46 (-11 – 103)	57.1 (7.3)	0.8
HL60	57 (-46 – 160)	44.9 (10.9)	-

Figure 4.2 Effect of Cladribine on MCF-7, MDAMB231 and HL60 Cell Viability

*A shows the concentration-response curve of the cell lines to cladribine in MCF-7, MDAMB231 and HL60 cell lines after 96 hours treatment. The table (B) gives the IC_{50} values (95% C.I.), the % response of control to cladribine at 0.1 μM (S.E.M) and the fold change in IC_{50} values compared to HL60 cells. Data are expressed as the mean of at least three independent experiments, repeated in triplicate. *($p<0.05$), **($p<0.01$) compared to MDAMB231 and HL60 cells. Data were analysed with one-way ANOVA and the Tukey-Kramer post hoc test. Closed symbols (ER-positive cells); open symbols (ER-negative cells).*

A



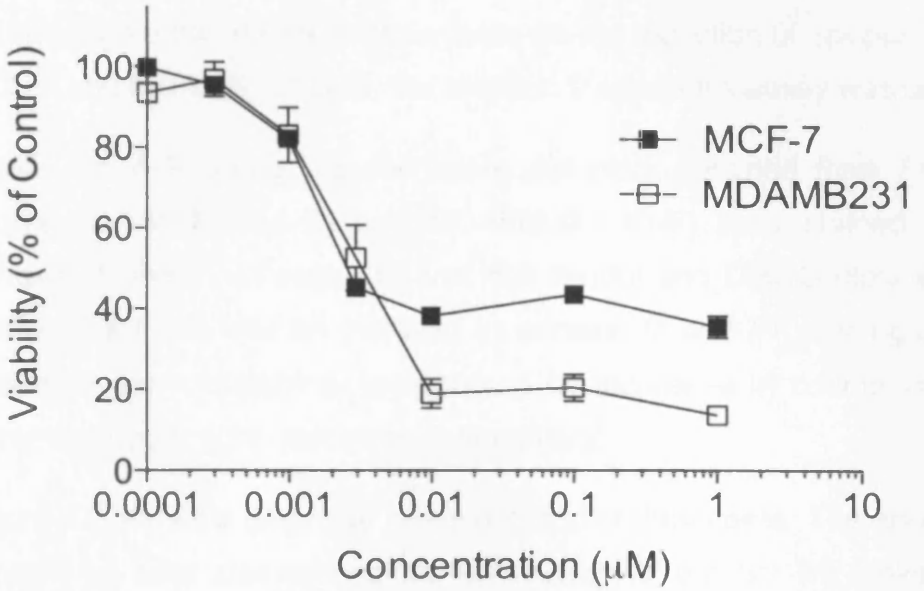
B

Cell Line	IC_{50} values (μM)	% Efficacy at 10 μM	IC_{50} value Change
MCF-7	13.6 (6.6 – 20.6)***	38.2 (4.0)***	103.1
MDAMB231	7.5 (4.0 – 11.1)**#	51.7 (3.2)***	57.1
HL60	1.3 (0.4 – 0.2)	99.0 (0.5)	-

Figure 4.3 Effect of Fludarabine on MCF-7, MDAMB231 and HL60 Cell Viability

*A shows the concentration-response curve of the cell lines to fludarabine in MCF-7, MDAMB231 and HL60 cell lines after 96 hours treatment. The table (B) gives the IC_{50} values (95% C.I.), the % response of control to fludarabine at 10 μM (S.E.M) and the fold change in IC_{50} values compared to HL60 cells. Data are expressed as the mean of at least three independent experiments, repeated in triplicate. **($p<0.01$), ***($p<0.001$) compared to HL60 cells. #($p<0.05$) compared to MCF-7 cells. Data were analysed with one-way ANOVA and the Tukey-Kramer post hoc test. Closed symbols (ER-positive cells); open symbols (ER-negative cells).*

A



B

Cell Line	IC_{50} values (nM)	% Efficacy at 1 μM
MCF-7	1.1 (0.8 – 1.3)	64.4 (2.7)**
MDAMB231	2.5 (-0.4 – 5.3)	86.3 (0.4)

Figure 4.4 Effect of Gemcitabine on MCF-7 and MDAMB231 Cell Viability

*A shows the concentration-response curve of cell lines to gemcitabine in MCF-7 and MDAMB231 cell lines after 96 hours treatment. The table (B) gives the IC_{50} values (95% C.I.) and the % response to gemcitabine at 1 μM (S.E.M). Data are expressed as the mean of at least three independent experiments, repeated in triplicate. **($p<0.01$) compared to MDAMB231 cells. Data analysed with a two-tailed Student's t test. Closed symbols (ER-positive cells); open symbols (ER-negative cells).*

4.4.5 Effects of clofarabine on induction of apoptosis in MCF-7 and MDAMB231 cell lines

To investigate the effects of clofarabine on the induction of apoptosis in MCF-7 and MDAMB231 cells, the annexin V apoptosis assay was used.

Figure 4.5 A-F shows representative dot plots obtained from FACS analysis of MCF-7 (A-C) and MDAMB231 (D-F) cells stained with annexin V and PI. In each cell line, the control and DMSO plots were similar, but there was an increase in annexin V and PI staining after treatment with clofarabine, represented by increases in counts in the lower and upper right quadrants, respectively.

Figure 4.6 shows a graphical representation of these data. The annexin V+ve/PI-ve cells stained positive for annexin V but not PI, typical of early apoptosis while the annexin V- and PI-positive cells are typical of late apoptosis or necrosis. After treatment with clofarabine, 18.0±2.8% and 9.7±1.7% cells were in early and late apoptosis in the MCF-7 cells, respectively, compared to 37.3±4.8% and 28.9±2.5% in the MDAMB231 cells, respectively. There were significant increases in both early apoptosis ($p<0.05$) and late apoptosis/necrosis ($p<0.001$) in MDAMB231 cells compared to MCF-7 cells.

4.4.6 Growth curves of breast cancer cell lines

The growth rates of the six breast cancer cell lines were investigated in order to determine if growth rate was correlated with activity of the PNA.

The MDAMB231 cells had the fastest growth rate followed by MCF-7 and T47D cells (Figure 4.7). The MDAMB436, BT 474 and SKBR-3 cells had reduced growth rates in comparison. No statistics were compiled as this study was carried out only once.

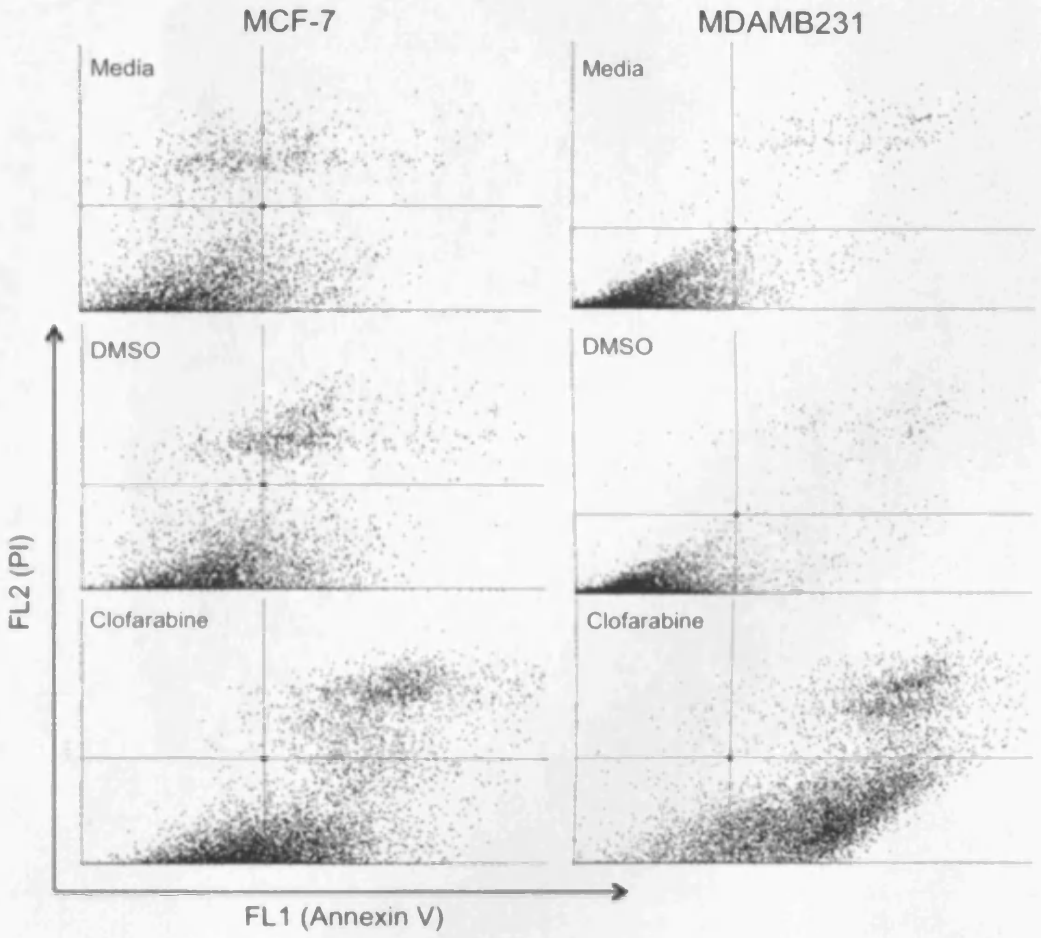


Figure 4.5 Dot Plots of Annexin V/PI Apoptosis Assay with FACS Analysis

These dot plots are representative examples (n = 3-4) of dot plots obtained from FACS analysis of annexin V/PI-stained MCF-7 (left) and MDAMB231 (right) cells treated with media alone (top), 0.05% DMSO (middle) or 1 μM clofarabine (bottom) for 96 hours. Log PI staining is on the FL2 channel (Y axis) and log annexin V staining is on the FL1 channel (X axis). Each plot shows 10,000 counts. Upper left quadrant = PI+ve/annexin V-ve; upper right quadrant = PI+ve/annexin V+ve; lower left quadrant = PI-ve/annexin V-ve; lower right quadrant = PI-ve/annexin V+ve.

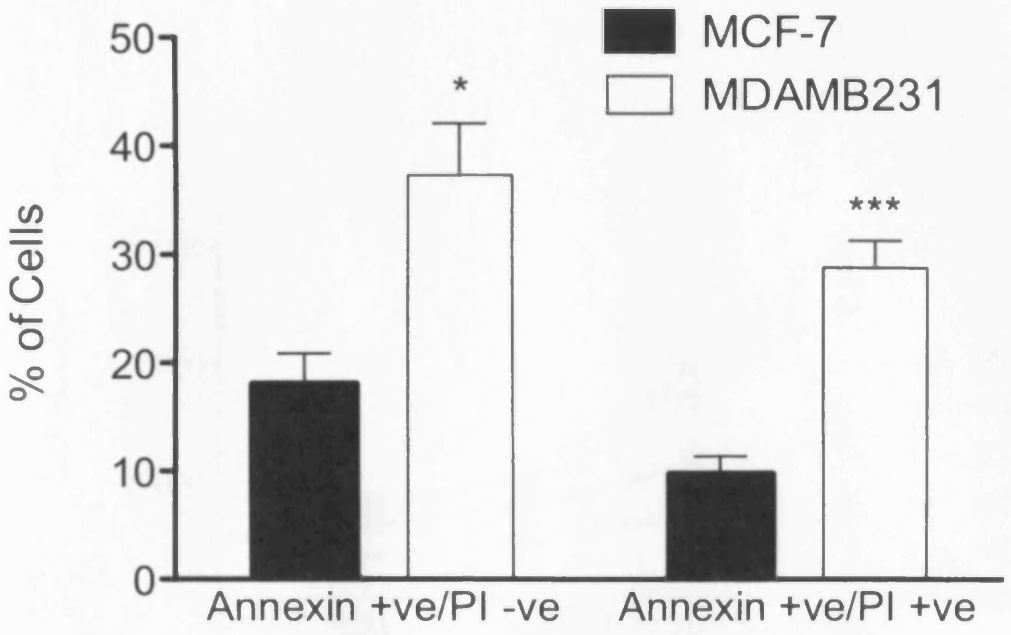


Figure 4.6 Annexin V/PI Apoptosis Assay with FACS Analysis on MCF-7 and MDAMB231 Cells After Treatment with 1 μM Clofarabine

*MCF-7 and MDAMB231 cells were treated with 1 μM clofarabine for 96 hours before staining with annexin V and PI and subsequent FACS analysis. Annexin +ve/PI-ve cells stained positive for annexin V but not PI. Annexin +ve/PI+ve cells stained positive for both annexin V and PI. The data were corrected for vehicle control (treated cells – DMSO vehicle) to correct for any false positives caused by the methodology. Data are expressed as the % of cells from 10,000 counts (n = 3-4) ± S.E.M. *($p<0.05$), ***($p<0.001$) compared to the MCF-7 cells analysed by a two-tailed Student's t test.*

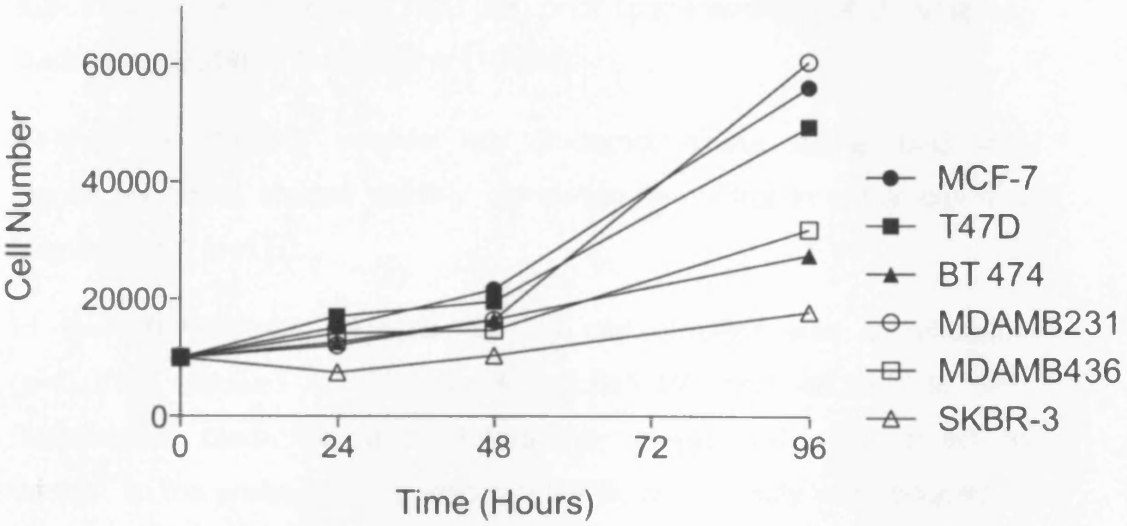


Figure 4.7 Cell Growth Assay of Breast Cancer Cells

Breast cancer cells, seeded at 10,000 cells per well in 24 plates were counted 24, 48 and 96 hours after seeding using a Beckman Z2 Coulter Counter. The data are expressed as the mean of triplicate wells from a single experiment and hence no error bars or statistics are presented. The ER-positive cell lines are depicted with closed symbols and ER-negative cell lines are represented by open symbols.

4.4.7 Effects of dCK saturation on the activity of PNAs

The action of PNA is dependent upon primary phosphorylation by dCK (Månsson *et al.* 1999). This was investigated in the MCF-7 and MDAMB231 cell lines by saturating the enzyme with its natural substrate, 2'-deoxycytidine (100 μM) prior to the addition of clofarabine, cladribine (1 μM) or fludarabine (10 μM).

Neither the DMSO vehicle nor 2'-deoxycytidine alone had any significant effect on cell viability compared to control in either cell line (Figure 4.8 A and B).

In the MCF-7 cells (Figure 4.8 A) cell viability was significantly ($p<0.001$) reduced to 57.1±4.1%, 42.8±1.1% and 48.8±0.5% with fludarabine, cladribine and clofarabine, respectively, compared to control. In the presence of 2'-deoxycytidine, cell viability was reduced to only 93.4±1.0%, 96.8±1.5% and 95.3±0.8% for fludarabine, cladribine and clofarabine, respectively, compared to control and these were significantly different to fludarabine, cladribine and clofarabine alone.

In the MDAMB231 cells (Figure 4.8 B) cell viability was significantly ($p<0.001$) reduced to 37.0±2.5%, 18.1±2.5% and 24.1±3.1% with fludarabine, cladribine and clofarabine, respectively, compared to control. In the presence of 2'-deoxycytidine, cell viability was reduced to 94.9±0.8%, 80.8±3.1% and 75.3±3.2% for fludarabine, cladribine and clofarabine, respectively. Fludarabine plus 2'-deoxycytidine was not significantly different compared to control, but cladribine and clofarabine plus 2'-deoxycytidine were significantly ($p<0.001$) reduced compared to control. In the presence of 2'-deoxycytidine, the activity of fludarabine, cladribine and clofarabine on cell viability was significantly reduced ($p<0.001$) compared to each drug alone.

The cladribine analogue 5'-deoxy-2'-deoxychloroadenosine (Figure 4.9 A) cannot be phosphorylated due to modification at C5 on the ribose

and is thus inactive. There was no significant effect of this drug compared to control at concentrations up to 100 μM (Figure 4.9 B).

4.4.8 Effects of hydroxyurea of PNA activity

The influence of the ribonucleotide reductase inhibitor, hydroxyurea (300 μM) on the efficacy of fludarabine (10 μM), cladribine (1 μM) and clofarabine (1 μM) was then investigated using the MTS assay.

The DMSO vehicle (0.05%) had no significant effect on cell viability in either cell line. Alone, hydroxyurea significantly ($p<0.001$) inhibited cell viability to 63.7±1.9% and 36.6±1.3% of control in the MCF-7 and MDAMB231 cells, respectively (Figure 4.10 A and B).

In the MCF-7 cells (Figure 4.10 A), cell viability was significantly reduced ($p<0.001$) compared to control by fludarabine (57.1±4.1%), cladribine (42.8±1.1%) and clofarabine (48.8±0.5%). In combination with hydroxyurea, viability was further reduced to 29.5±1.5%, 33.7±2.4% and 38.4±0.9% compared to control for fludarabine, cladribine and clofarabine, respectively. These values were significantly reduced compared to control ($p<0.001$), PNA ($p<0.05$) and hydroxyurea alone ($p<0.05$).

In the MDAMB231 cells (Figure 4.10 B), cell viability was significantly reduced ($p<0.001$) compared to control by fludarabine (36.1±3.1%), cladribine (18.1±2.5%) and clofarabine (24.1±3.1%). In combination with hydroxyurea, viability was further reduced to 23.2±0.4% by fludarabine, which was significantly reduced compared to control ($p<0.001$), fludarabine alone ($p<0.05$) and hydroxyurea alone ($p<0.05$).

In combination with hydroxyurea, no significant difference was observed in cell viability with cladribine (22.0±1.6% of control) or clofarabine (22.9±1.7% of control) compared to each drug alone. Cell viability was significantly reduced compared to control ($p<0.001$) and hydroxyurea alone ($p<0.05$).

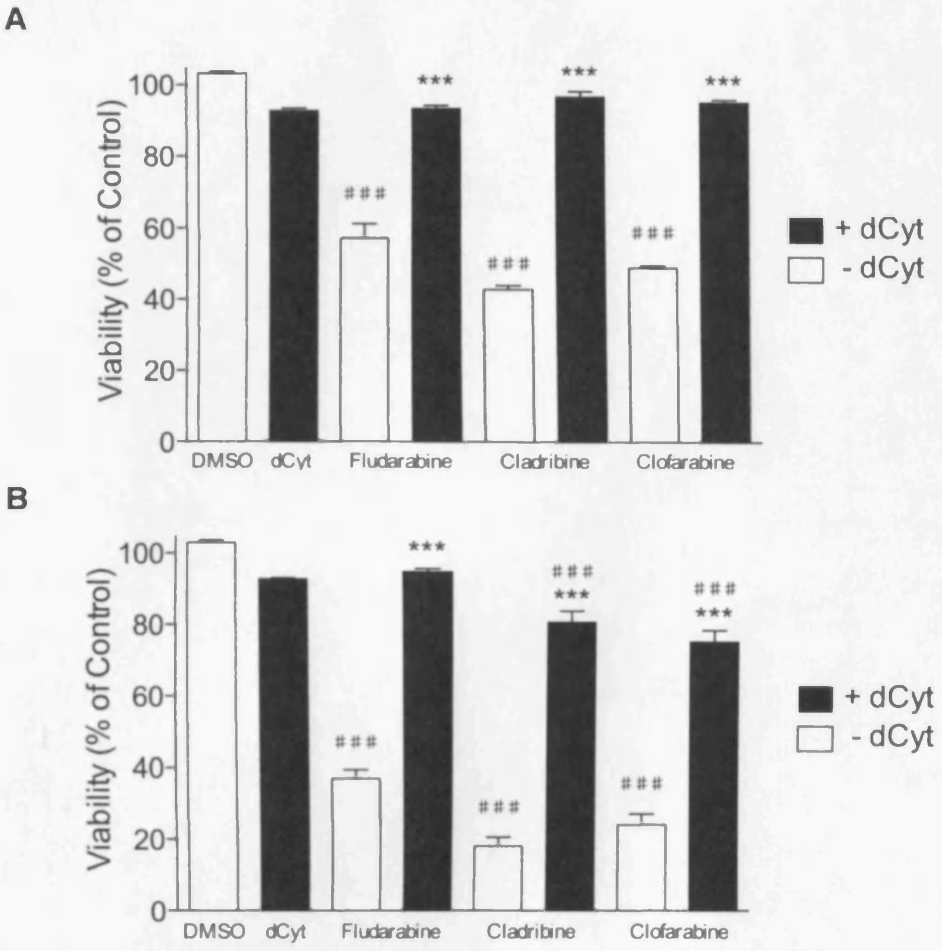
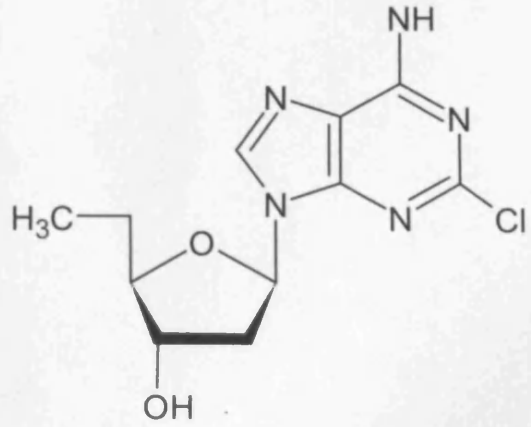


Figure 4.8 Effect of 2'-Deoxycytidine on PNA Activity

*The effects of 2'-deoxycytidine (dCyt; 100 μM) on the anti-cancer activity of fludarabine (10 μM), cladribine (1 μM) and clofarabine (1 μM) on MCF-7 (A) and MDAMB231 (B) breast cancer cells determined by the MTS assay. Data are expressed as % of viable cells compared to control and are the mean of three separate experiments, repeated in triplicate ± S.E.M. ***($p<0.001$) compared to respective NA alone; ###($p<0.001$) compared to control. Data were analysed with one-way ANOVA and the Tukey-Kramer post hoc test.*

A



B

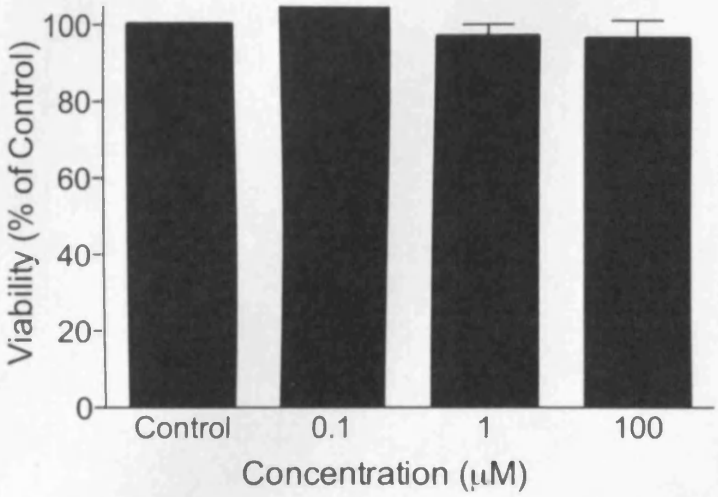


Figure 4.9 Effects of 5'-Deoxy-2'-Chlorodeoxyadenosine on MCF-7 Cell Viability

The effects (B) of the cladribine analogue, 5'-deoxy-2'-chlorodeoxyadenosine (A) on MCF-7 cell viability were determined by the MTS assay over 96 hours. Data are expressed as % of viable cells compared to control and are the mean of three separate experiments, repeated in triplicate ± S.E.M. No significant differences were observed.

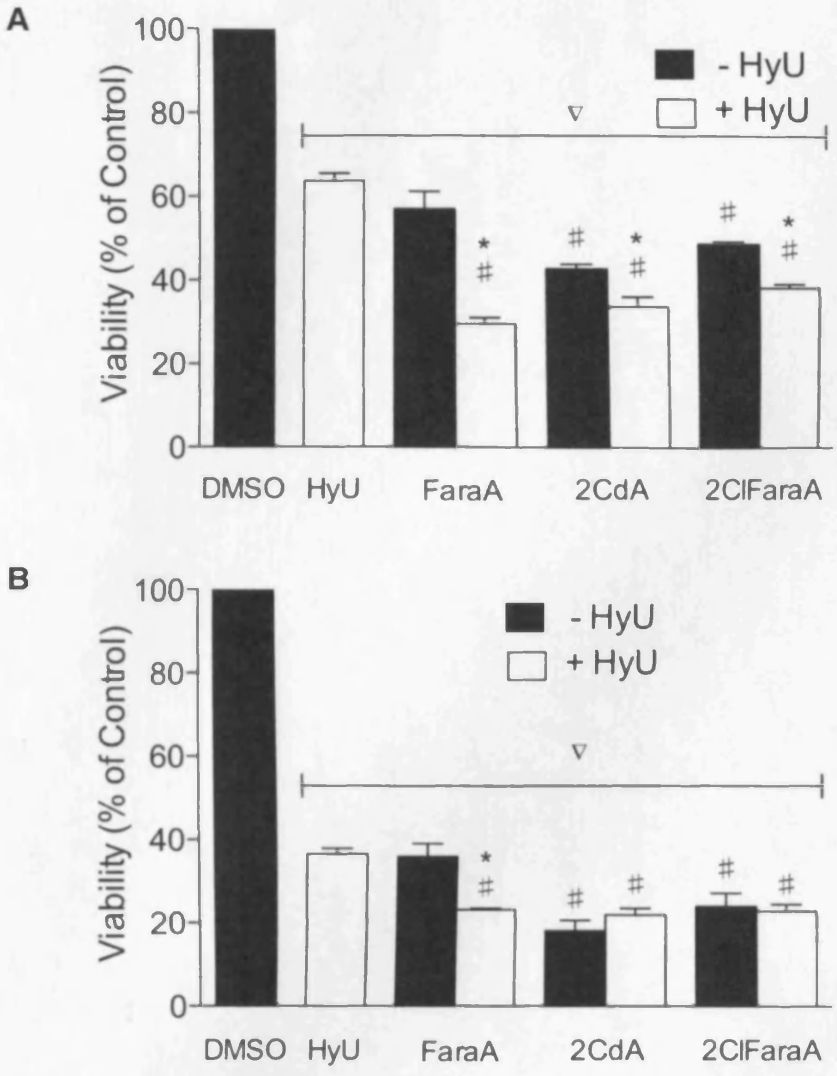


Figure 4.10 Effects of Hydroxyurea on PNA Drug Efficacy

*The effects of the ribonucleotide reductase inhibitor, hydroxyurea (HyU; 300 μM) on the efficacy of fludarabine (FaraA; 10 μM), cladribine (2CdA; 1 μM) and clofarabine (2ClFaraA; 1 μM) in MCF-7 (A) and MDAMB231 (B) cells determined by the MTS assay. Data are expressed as % of viable cells compared to control (media only) and are the mean of three separate experiments, repeated in triplicate ± S.E.M. *($p<0.05$) compared to respective NA alone; #($p<0.05$) compared to hydroxyurea alone. ∇($p<0.001$) compared to control. Data were analysed with one-way ANOVA and the Tukey-Kramer post hoc test.*

4.4.9 Effect of cladribine and clofarabine protides on cell viability

The effects of cladribine and clofarabine protides on cell viability were investigated in MCF-7 and MDAMB231 breast cancer cell lines. A description of the protide technology is given in section 4.5.9. The protide CdA1X is an analogue of cladribine, and ClFara1X and ClFara2Y are analogues of clofarabine.

In the MCF-7 cells (Figure 4.11 A), cladribine ($p<0.01$), clofarabine ($p<0.001$), ClFara1X ($p<0.001$) and ClFara2Y ($p<0.001$) at 1 μM significantly reduced cell viability compared to control. There was a significant reduction in the efficacy of CdA1X compared to cladribine ($p<0.01$), and ClFara1X compared to clofarabine ($p<0.001$). At 100 μM cladribine ($p<0.01$), CdA1X, clofarabine, ClFara1X and ClFara2Y ($p<0.001$) all significantly reduced cell viability compared to control. There was a significant improvement in CdA1X activity compared to cladribine ($p<0.05$) and significant increases in ClFara1X and ClFara2Y efficacies compared to clofarabine ($p<0.001$).

In the MDAMB231 cells (Figure 4.11 B), cladribine, clofarabine ($p<0.001$), ClFara1X, ClFara2Y ($p<0.01$) and CdA1X ($p<0.05$) at 1 μM all significantly reduced cell viability compared to control. At 100 μM, all the compounds significantly reduced ($p<0.001$) cell viability compared to control. There were significant decreases in the activity of CdA1X ($p<0.01$) and ClFara2Y ($p<0.05$) at 1 μM compared to their parent compounds. At 100 μM, CdA1X ($p<0.01$) and ClFara1X ($p<0.001$) and ClFara2Y ($p<0.01$) were all significantly more efficacious compared to their parent compounds.

4.4.10 Effects of clofarabine and cladribine protides over time

The effects of the protides on cell viability were studied over time to determine differential activity at different time points. Only one clofarabine protide was used, ClFara2Y, as this was the lead compound (personal communication, Prof. C. McGuigan).

In the MCF-7 cells at 1μM for each drug (Figure 4.12 A), CdA1X had significantly reduced activity at 72 ($p<0.05$) and 96 ($p<0.001$) hours, compared to cladribine. Only at 96 hours was cell viability significantly reduced compared to control with cladribine ($p<0.01$). The activity of ClFara2Y was significantly reduced compared to clofarabine at 24 ($p<0.05$) and 48 hours ($p<0.01$). Clofarabine reduced cell viability significantly at 24 ($p<0.01$), 48, 72 and 96 hours ($p<0.001$) compared to control. ClFara2Y reduced cell viability at 48 ($p<0.05$), 72 and 96 hours ($p<0.01$) compared to control.

In the MDAMB231 cell line at 1 μM (Figure 4.12 B) both cladribine and clofarabine significantly reduced cell viability at 48 ($p<0.01$), 72 and 96 hours ($p<0.001$) compared to control. CdA1X reduced cell viability significantly compared to control ($p<0.001$) at 96 hours and the reductions at 72 and 96 hours were significant ($p<0.001$) for ClFara2Y. At 48 ($p<0.01$), 72 and 96 hours ($p<0.001$) CdA1X activity was significantly less than that of cladribine.

At 100 μM, in the MCF-7 cells (Figure 4.13 A), both parent compounds ($p<0.05$ at 24 hours, $p<0.001$ at 48, 72 and 96 hours) and both protides significantly reduced cell viability at 24, 48, 72 and 96 ($p<0.001$) hours. At 24 hours, CdA1X was significantly better than cladribine at reducing cell viability compared to control ($p<0.001$). At all time points, ClFara2Y was significantly more active than clofarabine at reducing cell viability ($p<0.001$).

In the MDAMB231 cells (Figure 4.13 B), cladribine and CdA1X significantly reduced cell viability ($p<0.01$) compared to control at 48, 72 and 96 hours. Clofarabine reduced cell viability compared to control significantly at 48 ($p<0.05$), 72 and 96 hours ($p<0.001$) as did ClFara2Y at all time points ($p<0.001$). At 24 hours, ClFara2Y caused a significantly greater reduction in cell viability than clofarabine ($p<0.01$).

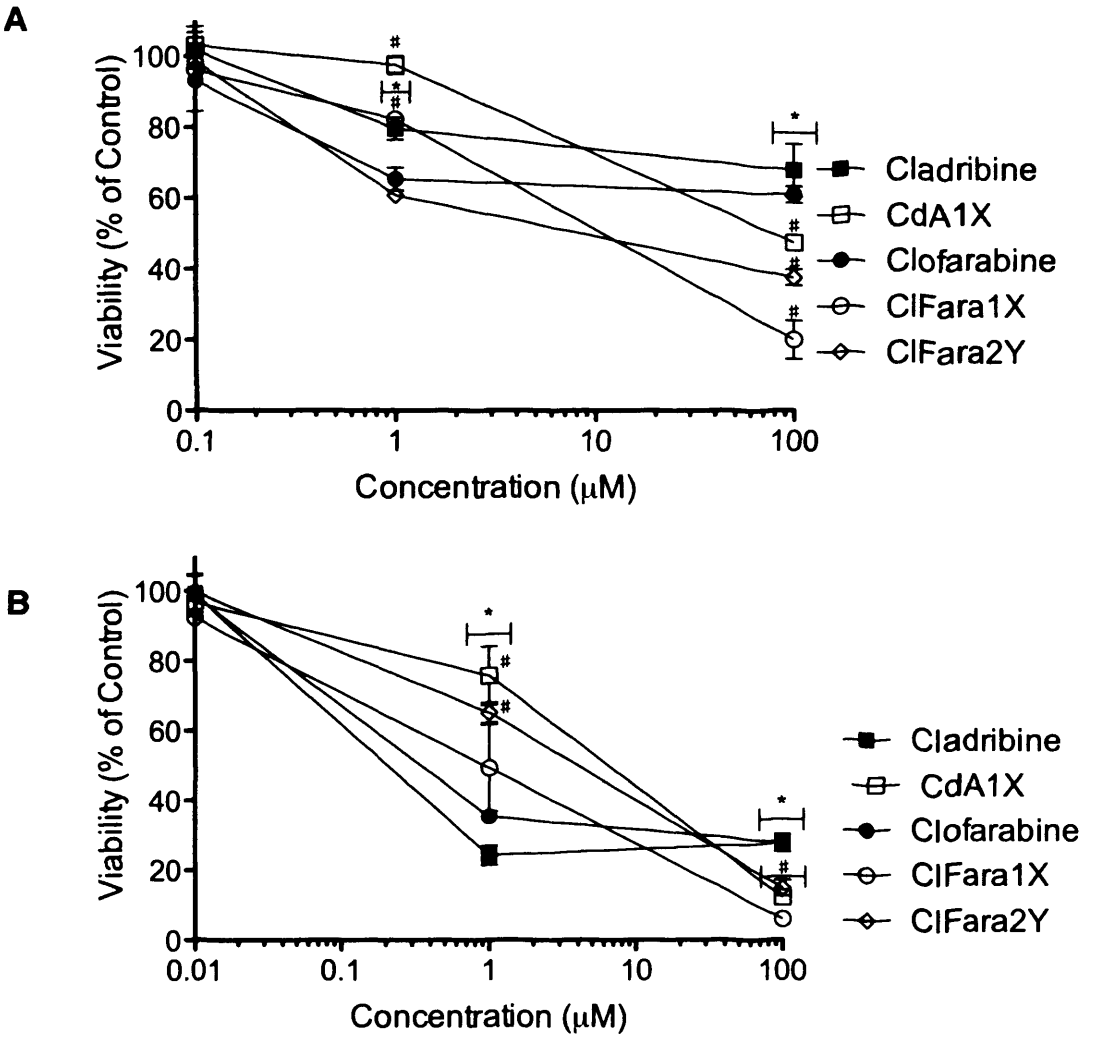


Figure 4.11 Effects of Cladribine and Clofarabine Protides on MCF-7 and MDAMB231 Cell Viability

*The effects on cell viability of a cladribine protide, CdA1X and two clofarabine protides, ClFara1X and ClFara2Y, were investigated over 96 hours using the MTS assay in MCF-7 (A) and MDAMB231 (B) cells. Data are expressed as % of viable cells compared to control and are the mean of three separate experiments, repeated in triplicate ± S.E.M. *($p<0.05$) compared to control; #($p<0.05$) compared to parent compound. The level of significance for each point was not included on the graph for clarity, but is included in the text (see section 4.4.9). Data were analysed using a one-way ANOVA and the Tukey-Kramer post hoc test.*

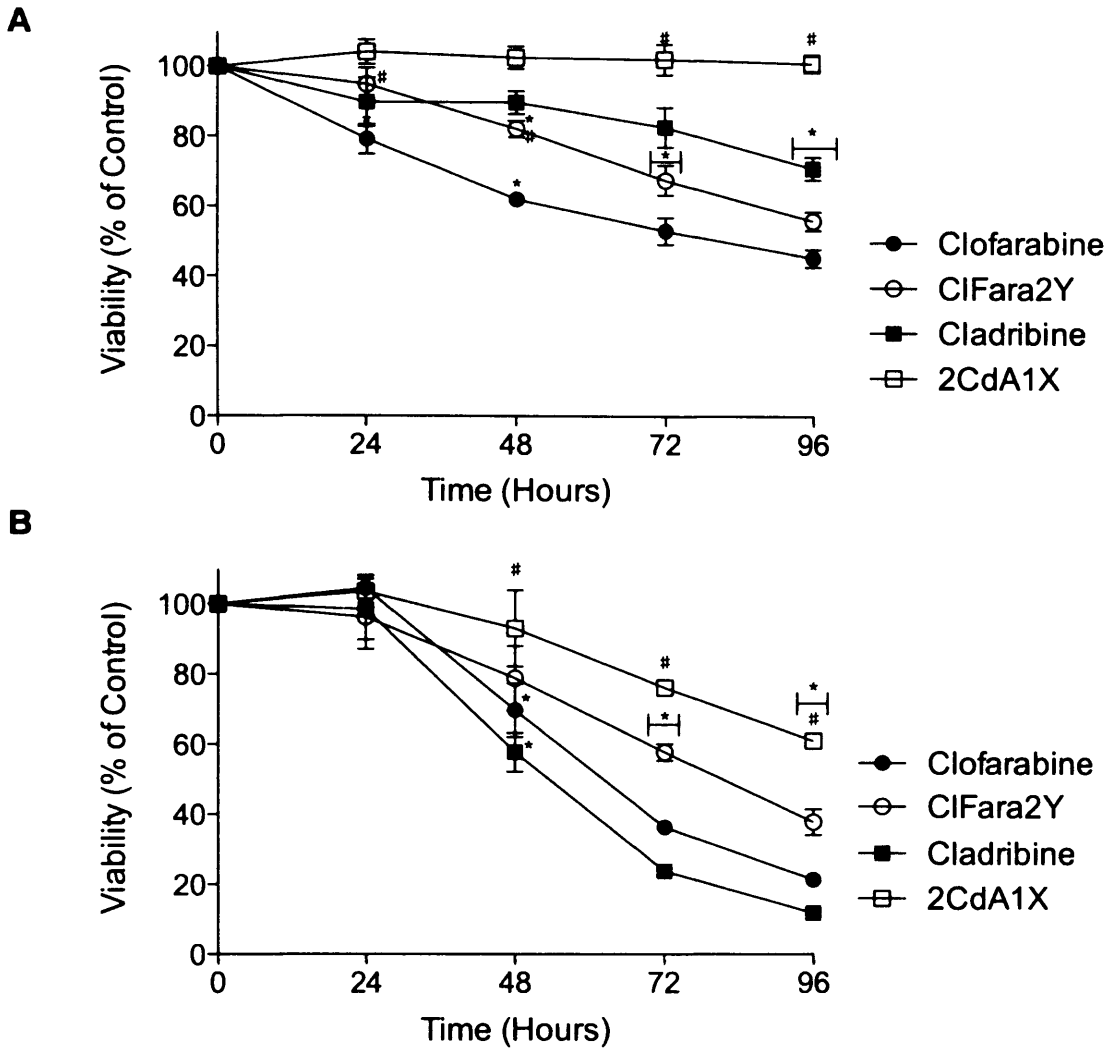


Figure 4.12 Effects of 1 μM Cladribine, Clofarabine, CdA1X and ClFara2Y on MCF-7 and MDAMB231 Cell Viability Over Time

*The effects of 1 μM cladribine, clofarabine and their respective protides, CdA1X and ClFara2Y, on MCF-7 (A) and MDAMB231 (B) cell viability were investigated at 24 hour time intervals using the MTS assay. Data are expressed as % of viable cells compared to control and are the mean of three separate experiments, repeated in triplicate ± S.E.M. *($p<0.05$) compared to control; $^{\sharp}$($p<0.05$) compared to parent compound. The level of significance was not included on the graph for clarity, but is included in the text (see section 4.4.10). Data were analysed using a one-way ANOVA and the Bonferroni post hoc test with selected pairs of data.*

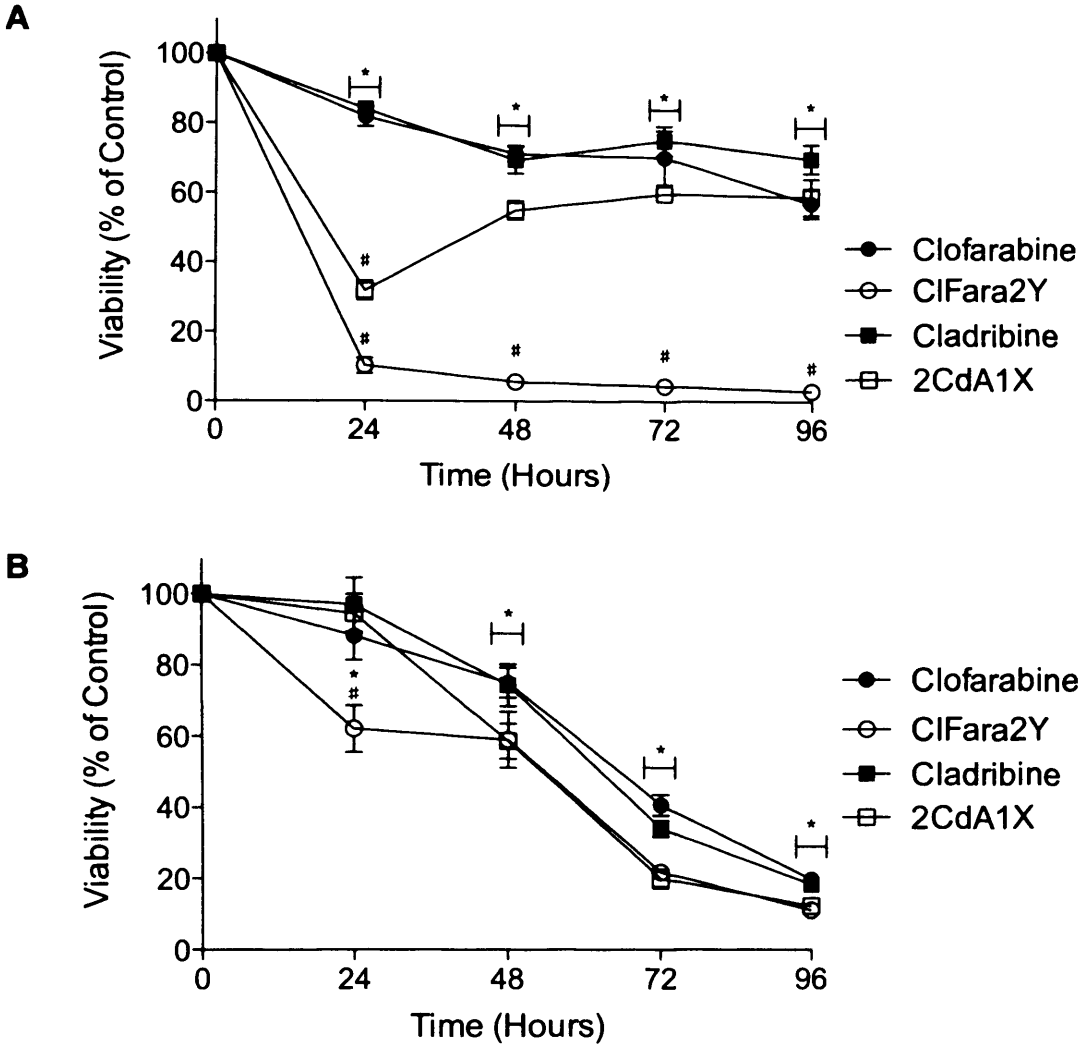


Figure 4.13 Effects of 100 µM Cladribine, Clofarabine, CdA1X and ClFara2Y on MCF-7 and MDAMB231 Cell Viability Over Time

*The effects of 100 µM cladribine, clofarabine and their respective protides, CdA1X and ClFara2Y, on MCF-7 (A) and MDAMB231 (B) cell viability were investigated at 24 hour time intervals using the MTS assay. Data are expressed as % of viable cells compared to control and are the mean of three separate experiments, repeated in triplicate ± S.E.M. *($p<0.05$) compared to control; #($p<0.05$) compared to parent compound. The level of significance was not included on the graph for clarity, but is included in the text (see section 4.4.10). Data were analysed using a one-way ANOVA and the Bonferroni post hoc test with selected pairs of data.*

4.5 Discussion

The aim of this chapter was to investigate the pharmacological actions of PNA on cell viability in a variety of human breast cancer cell lines. These effects were also examined in the HL60 leukaemia cell line as a control. The mechanisms of activation and action of these drugs were also investigated.

4.5.1 Molecular profile of breast cancer cell lines

The first part of this study was to determine which cell lines should be used to investigate the activity of the NA. A range of breast cancer cell lines were chosen to represent some of the most common molecular phenotypes of breast cancer seen clinically. Cells were chosen based upon their ER status primarily to encompass those that are ER-positive and ER–negative. Cell lines were also chosen based on whether they overexpressed Her2 and their p53 status. The molecular phenotype of each of the six breast cancer cell lines can be seen in Table 4.1.

The HL60 human promyelocytic leukaemia cell line was chosen to serve as a positive control for the NA studies as the effects of PNAs have been studied in these cells previously (Santini *et al.* 1996; Stachnik *et al.* 2005; Parker *et al.* 2009). These cells were chosen as they are a model of acute leukaemia, a disease which these drugs are used effectively to treat in the clinic (Robak and Wierzbowska 2009).

4.5.2 Clofarabine has a differential effect on cell viability

In the HL60 cells, clofarabine was shown to be the most effective of these drugs in reducing cell viability and fludarabine the least so, data which correspond with the current literature (Månsson *et al.* 1999; Bonate *et al.* 2006). Clofarabine was found to have an IC_{50} value of 20 nM, similar to previous results of 37-40 nM (Lotfi *et al.* 1999; Månsson *et al.* 1999). The IC_{50} values of cladribine and fludarabine were also similar to the literature, 57 nM compared with 41 nM and 1.3 μM

compared with 1.9 μM, respectively (Månsson *et al.* 1999). These data show that the PNA are working as expected and validate the experimental protocol.

The effects of clofarabine were investigated in the six breast cancer cell lines. The most striking effect observed was the significant differences in potency observed between ER-positive and ER-negative breast cancer cells. In ER-positive breast cancer cell lines, clofarabine had IC_{50} values of approximately 300 nM, compared to values of approximately 40 nM in the ER-negative cell lines. Whilst clofarabine was significantly less potent in these ER-positive cells compared to the HL60 cells, it was equally potent in the ER-negative cells compared to the HL60 cells. These data imply that clofarabine may have potential for clinical use in ER-negative breast cancers as less drug would be needed to produce a significant effect.

To further these studies, the efficiency of clofarabine to reduce cell viability at a concentration achievable clinically in plasma was determined. A concentration of 1 μM was chosen as clinical data suggested that, at the end of an infusion of the drug, clofarabine reached a median plasma concentration between 1 and 1.5 μM in patients treated with the maximal tolerated dose (Gandhi *et al.* 2003; Jeha *et al.* 2004; Faderl *et al.* 2005).

Clofarabine was equally efficacious at 1 μM in the ER-negative cell lines compared to the HL60 cells. This further suggests that clinically, clofarabine has potential for use against ER-negative breast tumours. In two ER-positive cell lines, MCF-7 and BT 474, clofarabine was significantly less efficacious compared to the HL60 cells at 1 μM. However, the efficacy of clofarabine in the T47D cells at 1 μM was equal to that observed in the ER-negative and HL60 cells. This difference is interesting because it means that whilst clofarabine is less potent in the T47D cells compared to the ER-negative cells, it is equally effective.

When discussing potency (IC_{50}) and efficacy at 1 μM, it is important to keep clear the distinctions between the two. The % efficacy at 1 μM is an important measurement as it suggests how the drugs might respond clinically at a concentration that could be achieved in patients. It gives an indication of how many of the tumour cells are likely to be killed by the drugs which is of clinical importance. However, this is not the maximal efficacy and is by definition, an 'arbitrary' unit at 1 μM. The potency (IC_{50} value) is a better indicator of the effects of these drugs *in vitro* as it takes into account the whole concentration-response curve. Therefore, this thesis will focus upon the potency of these drugs in this *in vitro* system and differences between cell lines will be discussed primarily as the differences in IC_{50} values.

These results support the concept that ER-negative tumours are more responsive to cytotoxic chemotherapy than ER-positive tumours, with TNBC being even more sensitive than Her2 overexpressing, ER-negative tumours (Gluz *et al.* 2009). Similarly, tumours belonging to the basal sub-type and those that are poorly differentiated and have a high proliferation index tend to be more sensitive to cytotoxic chemotherapy (Faneyte *et al.* 2003; Berry *et al.* 2006; Guarnari *et al.* 2006; Anders and Carey 2008). Various reasons have been proposed as to the cause of this, such as expression of P-glycoprotein, multi-drug resistance proteins and the bcl-2 proto-oncogene (Kerr 1995).

Overexpression of Her2 tends to predict a more aggressive phenotype, and this is associated with an increased *de novo* resistance to a standard chemotherapeutic regime of cyclophosphamide, methotrexate and 5'-fluorouracil (Stål *et al.* 1995; Pettitt *et al.* 2001). However, those Her2 overexpressing tumours show increased sensitivity to anthracycline, anthracycline plus taxane and doxorubicin therapy (Paik *et al.* 1998; Thor *et al.* 1998; Zhang and Liu 2008) compared to tumours with normal levels of Her2. Induction of Her2/ER cross-talk has been demonstrated to cause resistance to anti-hormone drugs previously

(Shou *et al.* 2004). There is no correlation with Her2 status and potency of clofarabine in these cell lines, however.

The tumour suppressor, p53, is often implicated in dictating chemotherapy efficacy. Galmarini *et al.* (2003a) found that p53-null colon cancer cells were more susceptible to the cytotoxic activity of cladribine compared to their p53 wild-type counterparts and suggested that the p53 status of a tumour dictated the apoptotic pathway induced. There was, however, no correlation observed here between p53 status and the effect of clofarabine, suggesting that p53 status and sensitivity to PNA in breast cancer cells does not correlate.

BRCA1 has also been demonstrated to have differential effects on chemotherapy responsiveness, depending upon the type of drug used (Quinn *et al.* 2003). However, of the six breast cancer cell lines tested here, only the MDAMB436 are reported to be BRCA1-mutated (Elstrodt *et al.* 2006) and so there was no obvious correlation between BRCA1 status and clofarabine potency.

4.5.3 Effects of cladribine and fludarabine on cell viability

The effects of cladribine in the MCF-7, MDAMB231 and HL60 cells were similar to that seen with clofarabine, albeit with a ~1.5-fold decrease in overall potency. These data suggest that cladribine and clofarabine may have similar mechanisms of action against these breast cancer cell lines, although clofarabine is more potent.

Fludarabine showed the least activity against MCF-7 and MDAMB231 cells when compared to cladribine and clofarabine. It also had less activity in the HL60 cells compared to the other two drugs. The reasons for this are unknown, but this result has been shown previously in the literature (Månsson *et al.* 1999; Marzo *et al.* 2001). This differential effect may be due to phosphorylation to the active phosphorylated compounds as cladribine and clofarabine are more efficiently phosphorylated (Gandhi *et al.* 2006; Harned and Gaynon 2008). This,

however, is unlikely as phosphorylation by dCK is not rate-limiting in the action of clofarabine and cladribine.

Alternatively, the differential potency of inhibition of ribonucleotide reductase or DNA polymerase may account for these effects. Clofarabine and cladribine are 10-fold more potent inhibitors (65 nM) of ribonucleotide reductase compared to fludarabine. Fludarabine is a more potent inhibitor of DNA polymerase-α (*Ki* = 0.7 μM) than cladribine and clofarabine (*Ki* = 3.5 and 4.7 μM, respectively). Fludarabine and cladribine are also relatively potent inhibitors of DNA polymerase-β (*Ki* = 5.8 and 42 μM, respectively) whereas clofarabine was less so (Parker et al. 1988; Parker *et al.* 1991; Xie and Plunkett 1995; Xie and Plunkett 1996; Parker *et al.* 1999; Gandhi *et al.* 2003; Larson and Venugopal 2009). These data therefore suggest that inhibition of DNA polymerase-α is less detrimental to the cells than inhibition of ribonucleotide reductase and depletion of dNTP pools.

Unlike the effects seen with cladribine and clofarabine, there was a highly significant difference between the effects of fludarabine in the MDAMB231 cells compared with the HL60 cells. This further suggests a different mechanism of action of fludarabine compared to cladribine and clofarabine and may also indicate that the HL60 cells are more sensitive to inhibition of DNA polymerase-α than the MDAMB231 cells.

Alternatively, clofarabine has been shown to alter the transmembrane potential of mitochondria and release of mitochondrial apoptotic factors and this was not seen with fludarabine. This may explain the effects on cell viability observed (Carson *et al.* 1992; Genini *et al.* 2000a; Péréz-Galen *et al.* 2002; Bonate *et al.* 2006). However, Klöpfer *et al.* (2004) stated that both cladribine and fludarabine are able to induce apoptosis by a mitochondrial cell death pathway and so this is less likely.

Clofarabine boasts the most clinical potential compared to cladribine and fludarabine being both a potent inhibitor of ribonucleotide reductase

and DNA polymerase (Tseng *et al.* 1982; Parker *et al.* 1991). Physiologically, besides being resistant to degradation by ADA, it is also the most acid-stable compound, the most lipophilic and is also resistant to bacterial nucleoside phosphorylase (Zhenchuk *et al.* 2009). Clofarabine also has a slower elimination rate compared with cladribine. These features suggest it is more stable in the body and less likely to be broken down to toxic metabolites like 2-chloroadenine, therefore, reducing side effects, and increasing its duration of action. Based upon the data here, and the beneficial properties of clofarabine over cladribine and fludarabine clinically, clofarabine was chosen to study in more depth throughout the rest of this thesis.

4.5.4 Effect of gemcitabine on cell viability

Gemcitabine is a pyrimidine NA and, in combination with paclitaxel, has recently been approved for the treatment of metastatic breast cancer in patients who have relapsed and had prior adjuvant/neo-adjuvant chemotherapy (NICE guidelines, 2007). Its activity against the MCF-7 and MDAMB231 cells was investigated to compare with the PNA *in vitro*.

Interestingly, gemcitabine had IC_{50} values of 1.1 and 2.5 nM in the MCF-7 and MDAMB231 cells, respectively. This is a significant increase (MCF-7 $p<0.01$ and MDAMB231 $p<0.05$) in the activity of gemcitabine compared to the clofarabine by 300- and 16-fold in the MCF-7 and MDAMB231 cells, respectively.

Unlike pyrimidine analogues, the rate limiting-step for clofarabine and cladribine activity is the conversion of the monophosphate to the diphosphate, and not primary phosphorylation by dCK (Gandhi *et al.* 2003). The differences observed between clofarabine and gemcitabine may therefore be a consequence of high expression of dCK in these cell lines. Similarly, gemcitabine is more potent inhibitor or the various

subunits of ribonucleotide reductase than PNA and this may account for the increased potency.

Despite the potency differences between gemcitabine and clofarabine, clofarabine should not be overlooked as a potential treatment of breast cancer. Gemcitabine is rapidly deactivated by cytidine deaminase and has a very short *in vivo* half-life of 8 minutes (Abbruzzese *et al.* 1991). In order to accumulate intracellular gemcitabine triphosphate, plasma concentrations of gemcitabine must be ≥10μM (Zucchetti *et al.* 2004).

Deoxycytidine triphosphate and gemcitabine triphosphate (dFdCTP) are negative feedback regulators of dCK. Concentrations of these metabolites of 25 μM can cause greater than 90% inhibition of the primary phosphorylation of gemcitabine by dCK (Gandhi and Plunkett 1990; Bouffard *et al.* 1993; Veltkamp *et al.* 2008) and may explain why dFdCTP formation is saturated at plasma concentrations in a similar range *in vivo* (15-20 μM) (Abbruzzese *et al* 1991).

Taken together, these data suggest that clinically plasma concentrations in the range of 10 – 20 μM are optimal for accumulation of the active metabolite of gemcitabine, dFdCTP. The data herein suggest that at 1 μM there was no significant cytotoxic difference observed between gemcitabine and clofarabine in the MDAMB231 cells. Therefore, although gemcitabine may have better *in vitro* potency, *in vivo*, clofarabine may be just as effective as gemcitabine. This data may be clinically relevant regarding *de novo* and acquired resistance to gemcitabine. Resistance to gemcitabine has been suggested to occur via dCK deficiency (Jordheim *et al.* 2006; Ohhashi *et al.* 2008), but the data in this thesis suggest that clofarabine activity is not defined by dCK levels, opening the door for clofarabine in the event of gemcitabine resistance (see Chapter 5). Similarly it has also been demonstrated that cells resistant to cladribine were not resistant to gemcitabine or cytarabine (Schirmer *et al.* 1998) further suggesting that cross-resistance may not occur.

4.5.5 Clofarabine induces apoptosis in MCF-7 and MDAMB231 cells

To determine if clofarabine was causing apoptosis, flow cytometry with annexin V and propidium iodide staining was performed. After treatment with 1 μM clofarabine for 96 hours, there was an increase in the amount of annexin V staining in both MCF-7 and MDAMB231 cell lines compared to media when normalised to 0.05% DMSO vehicle control. This indicates that clofarabine can cause apoptosis in both these cell lines, confirming the cytotoxic nature of clofarabine against cancer cells. Numerous studies have shown this, albeit in leukaemia cell lines (Leoni *et al.* 1998; Genini *et al.* 2000a; Genini *et al.* 2000b; Takahashi *et al.* 2002; Bonate *et al.* 2006; Zhang *et al.* 2009). This is the first example of clofarabine inducing apoptosis in breast cancer cells at clinically relevant concentrations. Hashemi *et al.* (2003; 2004) suggested that cladribine was able to cause apoptosis in MCF-7 and MDAMB436 cells, respectively, but this was only demonstrated at concentrations >25 μM that are not clinically relevant.

Clofarabine was shown to cause a significant increase in both early and late apoptotic cells in the MDAMB231 cells, compared to the MCF-7. These data further confirm the MTS results, suggesting that clofarabine is more active in the MDAMB231 cells compared to the MCF-7 cells. It would also be interesting to see if similar differences were observed in the other breast cancer and HL60 cell lines to confirm the MTS data.

4.5.6 The growth rate of breast cancer cells does not influence PNA potency

The differences in the activity of the PNA in the different breast cancer cell lines could be due to their rate of proliferation as cells that are undergoing more rapid division will have an increased need for DNA synthesis. This was investigated by determining the proliferation rates of each of the six breast cancer cell lines in preliminary experiments.

It can clearly be seen that there was no particular correlation observed between growth rate and the activity of the NA cladribine and clofarabine. The MDAMB231 cells were the fastest growing, and the ER-negative, Her2 over-expressed SKBR-3 cells were the slowest. The MCF-7 and T47D cells grew at a slightly reduced rate compared to the MDAMB231 cells, but were both faster than the BT 474, MDAMB436 and SKBR-3 cells.

4.5.7 PNA require phosphorylation by dCK for their activity

PNA are prodrugs that require intracellular phosphorylation by a nucleoside kinase to become active. The main enzyme responsible for this reaction for both purine and pyrimidine analogues is dCK (Lotfi *et al.* 1999; Parker *et al.* 1999; Månsson *et al.* 2003; van der Wilt *et al.* 2003). dCK is a rate-limiting enzyme in the nucleoside salvage pathway as it catalyses the first phosphorylation reaction with 2'-deoxycytidine, its natural substrate (Hatzis *et al.* 1998) although it can also phosphorylate both 2'-deoxyadenosine and 2'-deoxyguanosine (van der Wilt *et al.* 2003). Thymidine kinase and deoxyguanosine kinase have also been implicated in the primary phosphorylation of NA, although to a significantly lesser extent (Månsson *et al.* 1999; van der Wilt *et al.* 2003).

There is no specific inhibitor of dCK but Bilgeri *et al.* (1993) and Joachims *et al.* (2008) were able to show that micromolar concentrations of the natural substrate, 2'-deoxycytidine, could competitively inhibit the activity of dCK *in vitro*. For this study an increased concentration of 100 μM of 2'-deoxycytidine was used compared to the 50 μM in the previous study by Joachims *et al.* (2008) on murine thymocytes. Cancer cells tend to have increased levels of dCK, so an increased concentration was used to account for this (van der Wilt *et al.* 2003).

In both the MCF-7 and MDAMB231 cells, pre-treatment with 2'-deoxycytidine significantly reduced the action of all three purine analogues compared to the activity of the analogues alone. In the MCF-7 cells there was no significant difference observed between control, 2'-deoxycytidine alone and drug plus 2'-deoxycytidine, suggesting that dCK is the sole enzyme involved in activation of these drugs. However, in the MDAMB231 cells, in the presence of 2'-deoxycytidine, there was still a significant reduction in cell viability with cladribine and clofarabine, but not for fludarabine. This suggests that fludarabine is solely phosphorylated by dCK, but other enzymes may be involved in the phosphorylation of cladribine and clofarabine. However, this involvement is likely small due to the minor differences observed. This could be due to thymidine or deoxyguanosine kinase (Månsson *et al.* 1999; van der Wilt *et al.* 2003).

An analogue of cladribine, 5'-deoxy-2'-chlorodeoxyadenosine, (a generous gift from Professor Chris McGuigan, Welsh School of Pharmacy) was used to further these studies. This analogue, has the hydroxyl replaced at the 5' position on the sugar moiety with a methyl group (see Figure 4.9 A). The substitution impairs the ability of this molecule to be phosphorylated at this position, rendering it a nucleoside. As the action of cladribine and other analogues is dependent upon phosphorylation, this drug should be inactive against cells. Even at 100 μM, 5'-deoxy-2'-chlorodeoxyadenosine had no significant effect on cell viability in the MCF-7 cells. These data show further that cladribine at least, must be phosphorylated at the 5' position to be active.

4.5.8 Hydroxyurea influences the action of PNAs

Hydroxyurea is a clinically used ribonucleotide reductase inhibitor that works by scavenging the tyrosyl free radical essential for redox found on the RRM2 and p53R2 subunit (Sneeden and Loeb 2004). It has been shown to inhibit the growth of cancer cells both *in vitro* and *in vivo*

(Juul *et al.* 2010). It has also been shown to increase the efficacy of NA by decreasing intracellular dNTP pools allowing a greater accumulation of NA into DNA (Sato *et al.* 1984; Bhalla *et al.* 1991; Kano *et al.* 2000; Zhou *et al.* 2002; Karp *et al.* 2008). The effects of hydroxyurea were investigated in combination with PNA to examine potential for combination treatment and to help elucidate pharmacologically the role of ribonucleotide reductase in PNA drug activity in these cells.

A concentration of 300 μM hydroxyurea was used as this was found to be the IC_{50} value (Yang *et al.* 2001; Rajwade *et al.* 2009) and has been used previously to inhibit the actions of the RRM2 subunit and ribonucleotide reductase activity *in vitro* (Kuo *et al.* 2003).

In both MCF-7 and MDAMB231 cells, hydroxyurea inhibited cell viability by 40 and 60% compared to control, respectively, meaning that inhibition of ribonucleotide reductase reduces cell survival significantly. The significant difference ($p<0.001$; two-tailed Student's *t* test) between the two cell lines could be explained by an increase in ribonucleotide reductase RRM2 or p53R2 subunit expression in the MCF-7 cells compared to the MDAMB231 cells and has been suggested before as a mechanism of resistance to hydroxyurea (Zhang *et al.* 2009).

In combination with hydroxyurea, fludarabine significantly decreased cell viability compared to fludarabine or hydroxyurea alone by 30 and 15% in the MCF-7 and MDAMB231 cells, respectively. This is probably an additive effect rather than synergistic effect and has been suggested previously (Sato *et al.* 1984; Kano *et al.* 2000). It is likely that the decrease in free dNTP pools by hydroxyurea encourages fludarabine incorporation into DNA leading to strand breakage and cell death (Bhalla *et al.* 1991; Kano *et al.* 2000; Zhou *et al.* 2002; Karp *et al.* 2008). It also suggests that fludarabine primarily works by a mechanism independent of inhibition of ribonucleotide reductase

In the MCF-7 cells, there was a small but significant increase in efficacy of cladribine and clofarabine in combination with hydroxyurea compared to each analogue alone, although this increase in efficacy was 3-fold lower than that observed with fludarabine. There was no difference observed between these groups in the MDAMB231 cells. These data imply that cladribine and clofarabine are primarily working via inhibition of ribonucleotide reductase, and this could be due to their more potent inhibition of this enzyme than fludarabine (Parker *et al.* 1991). As cladribine and clofarabine were still having some effect in the MCF-7 cells, this could be explained by an increase in ribonucleotide reductase activity in these cells, and so is not fully inhibited by hydroxyurea. Alternatively hydroxyurea may be having a non-specific effect and has been shown to inhibit ERK and JNK signalling, inhibits the action of catalase and is known to influence iron uptake and expression of transferrin receptors (Chitamber and Wereley 1995; Park *et al.* 2001; Juul *et al.* 2010).

These data with hydroxyurea should be interpreted with caution as the effects seen may not be due to inhibition of p53R2 or RRM2. To confirm these studies, investigating changes in the dNTP pool would help identify a ribonucleotide reductase-specific mechanism. Similarly, the use of a different inhibitor would be useful. Triapine is a new ribonucleotide reductase inhibitor that works in the same way as hydroxyurea, but shows vastly improved specificity and has an IC_{50} value of 112 and 144 nM for p53R2 and RRM2, respectively (Shao *et al.* 2004; Karp *et al.* 2008). The fact that hydroxyurea is producing such pronounced effects alone may be skewing the data in combination with the NA.

4.5.9 Effects of protides on cell viability

Nucleoside prodrugs represent a novel way of delivering NA drugs and have potential as new therapeutics. Their aim is to by-pass the rate-limiting and adverse steps in the parent NA modifications, with the

ultimate result of increased or new activity against cancers and viruses (Wagner *et al.* 2005). There was a two-fold aim to using prodrugs in this thesis; to establish if prodrugs of the NA have potential as new therapeutics against breast cancer and secondly, to help elucidate mechanism(s) of action of the parent compounds in breast cancer cells *in vitro*. For intellectual property reasons neither the structures or specific details of any of the prodrugs can be disclosed in this thesis. The prodrugs used in this thesis are based upon phosphoramidate technology where the protective groups are amides or esters and for consistency will be called protides throughout.

Protides are monophosphates of the parent nucleoside and therefore can benefit from bypassing primary phosphorylation by dCK (Congiatu *et al.* 2005). Although the rate-limiting step is not primary phosphorylation by dCK for PNA (Xie and Plunkett 1995; Albertioni *et al.* 1998; Lotfi *et al.* 1999), it is still a necessary step. As the nucleotide cannot pass through the plasma membrane due to its charge, protecting groups are added to mask this charge and allow it to diffuse freely through the membrane. The protecting groups are then cleaved off inside the cell by cellular esterases and amidases to reveal the nucleotide monophosphate. The generic structure of a protide of cladribine is shown in Figure 4.14.

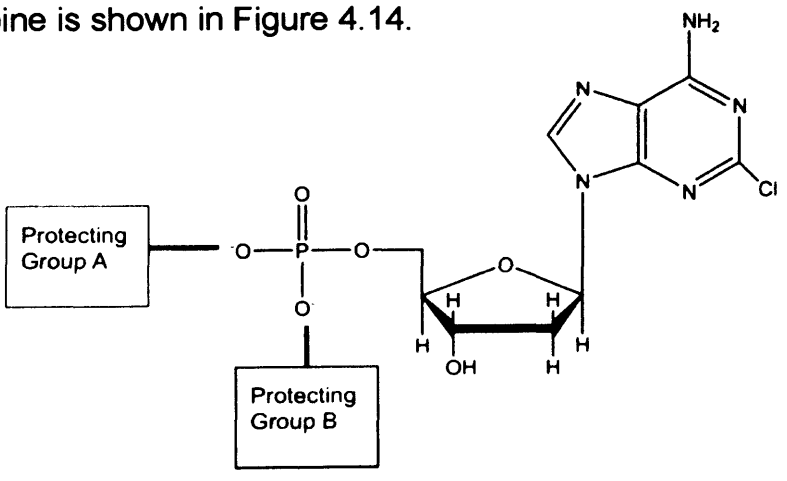


Figure 4.14 Generic Structure of a Cladribine Protide

Concentration-response curves for the various protides and the parent compounds were investigated including an inactive concentration of the parent compounds (0.1 and 0.01 μM for the MCF-7 and MDAMB231 cells, respectively). In both cell lines, and for all the protides, none had any significant effect on cell viability at these lowest concentrations. Only at 100 μM were the protides significantly better than the parent compounds in both cell lines. Importantly, at clinically achievable concentrations of cladribine (0.1 μM) and clofarabine (1 μM), there was no significant improvement in the activity of the protides. This is possibly due to low levels of esterases or amidases to cleave the protecting groups and reveal the nucleotide.

These data strongly suggest that these protides are unlikely to have clinical potential as against breast cancer. A key features of NA to support their clinical use is their selectivity for cancerous cells due to the increased levels of dCK in cancer cells compared with normal cells (Eriksson *et al.* 1994; van der Wilt *et al.* 2003; Hubeek *et al.* 2005). By by-passing this primary phosphorylation they lose this selective feature which could lead to increased effects on normal tissue, therefore increasing side effects for the patient. Similarly, by being able to diffuse through the membrane, the expression of NT are less important for the protides. Malignant cells have been identified as having increased levels of NT compared to normal cells, which may help to explain the increased sensitivity of these cells to NA (Mackey *et al.* 1998; Pennycooke *et al.* 2001; Zhang *et al.* 2007). Again by-passing this, the protides lose another selective feature that may increase side effects.

In help determine the mechanism(s) of action of the parent NA, time course profiles were set up with these drugs and the protides. It was hypothesised that the protides may work faster than the parent compounds, regardless of their efficacy at 96 hours. Cell viability was determined at 24 hour intervals for clofarabine, cladribine and two protides, CdA1X and ClFara2Y at 1 μM (a clinically relevant

concentration) and at 100 μM as this was the only concentration that the protides had any beneficial effect over the parent compounds.

In both the MCF-7 and MDAMB231 cell lines, there was no improvement observed with either protide when compared to their respective parent compound at 1 μM at any time interval, and they were significantly less effective at various time points. These effects could be due to low expression of cellular esterases or amidases. What these data highlight is that neither cellular entry nor conversion to the monophosphate are rate-limiting steps in the activity of cladribine or clofarabine in the MCF-7 or MDAMB231 cell lines.

When the effects of the protides were studied at 100 μM, ClFara2Y and CdA1X resulted in a 90 and 70% reduction in cell viability, respectively, after 24 hours in the MCF-7 cells. Compared to the parent compounds, this is significantly greater. After 96 hours treatment with ClFara2Y, there was no significant alteration in cell viability compared to 24 hours. This was expected as nearly all of the cells were dead after only 24 hours. However, after 48 hours treatment with CdA1X, there was a 20% increase in the number of viable cells compared to 24 hours. This suggests that the MCF-7 cells are able to recover somewhat after the initial insult.

The reason for this is unknown, but it is possible that the protide oligophosphates accumulate more rapidly than their parent compounds resulting in rapid cell death. Whereas with the parent compounds, the cells can clear or deactivate the cytotoxic oligophosphate before they build up to a significantly cytotoxic level due to their slow accumulation.

Alternatively, repair of the DNA insult may be able to occur with the parent compounds, but the rapid accumulation of toxic oligophosphates from the protides may be too quick for DNA repair process to begin. The wild-type p53 in MCF-7 cells may support this argument. Furthermore, the recovery of the MCF-7 cells at 48 hours after CdA1X

treatment also suggests an adaptive feature of the MCF-7 cells after the initial insult that could be repair of the DNA. As the MDAMB231 cells have mutated p53 and mutated DNA repair mechanism(s), the rate of accumulation of cytotoxic oligophosphates is perhaps less relevant and may explain why even at higher concentrations of the drugs, there was no increase in effect as the effects are already saturated.

The dramatic cytotoxic effects seen in the MCF-7 cells at high concentrations of the protides could be due to accumulation of the protecting groups cleaved from the protide to unmask the nucleotide. Although it cannot be stated what these groups are, literature searches have revealed that high concentrations of these metabolites can cause cytotoxicity in a variety of cancer cell lines in culture. This could be investigated by treating the cells with these metabolites and measuring their cellular accumulation with HPLC, and their cytotoxicity with viability assays.

In the time course experiments the significant difference at 100 μM between the clofarabine protides and clofarabine was not observed as before (section 4.4.8). This is probably due to inter-experimental variability, as there is only an 8% difference between the results at 96 hours in section 4.4.9 and 4.4.10. These data suggest that, in the MDAMB231 cells, increasing the concentration of protide or parent analogue is not going to increase the anti-cancer activity of either compound dramatically over shorter time periods than 96 hours. This could be because at 1 μM either the activation enzymes, monophosphate or diphosphate purine kinases, and/or the cellular targets of these oligophosphates are saturated.

4.6 Conclusion

The aim of this chapter was to investigate the activity and action of PNA in human breast cancer cell lines *in vitro*.

Clofarabine was the most potent of the three PNA tested in breast and leukaemia cell lines. It was also more potent in ER-negative compared to ER-positive cell lines, and its potency in these ER-negative cells was comparable to the activity seen in the HL60 leukaemia cell line. Cytotoxic activity of the PNA is also dependent upon phosphorylation by dCK. These data indicate the potential for the use of clofarabine in ER-negative breast cancers.

The differences in potency of clofarabine between the ER-negative and ER-positive cells was not addressed in this chapter. However, based upon the pharmacological profiles of the other PNA and the ribonucleotide reductase inhibitor, hydroxyurea, differences in the expression and activity of ribonucleotide reductase may be a plausible explanation. Similarly, the differential effects were not attributed to the rate of proliferation or different molecular phenotypes of these cells, besides ER status.

Results obtained with clofarabine and cladribine protides indicated that these novel agents are unlikely to be of clinical use but did, however, suggest that neither intracellular phosphorylation to the monophosphate, nor cellular entry are rate-limiting steps in the effectiveness of clofarabine or cladribine.

The differences observed between ER-positive and ER-negative cells raised further questions that are addressed in the next chapter. Here, studies were carried out to try and identify the causes of this increased resistance in the ER-positive cells using molecular biological techniques, with particular focus on the MCF-7 and MDAMB231 cells.

Chapter 5: Mechanisms of Increased Resistance to Clofarabine in ER-Positive Breast Cancer

5.1 Introduction

In the previous chapter, a correlation between ER status and the potency of PNAs were identified. Although still sensitive to clofarabine at clinically relevant concentrations (~300 nM), the ER-positive tumours were 8-fold more resistant to the action of clofarabine than the ER-negative tumours. PNA have not been widely studied in solid tumours and so there is no data on mechanisms of resistance to these drugs in solid tumour cell lines. There are, however, a large number of studies that have looked at the resistance of PNA in leukaemia and other cell lines of haematological malignancies. The vast majority of these data look at acquired resistance to the drugs rather than *de novo* resistance as is the case here, and again means that direct comparisons are difficult to make. These studies are useful, however, as they suggest rational mechanisms and targets that could be identified in solid tumours.

Three proposed routes of resistance to PNA have been suggested in the literature (Galmarini *et al.* 2001);

1. Insufficient accumulation of the active triphosphate due to
    a. decreased dCK expression
    b. increased expression of cN-II
    c. decreased nucleoside influx, or increased efflux.
2. Decreased ability of the triphosphate to affect DNA or dNTP pools from
    a. increased/altered ribonucleotide reductase expression
    b. altered DNA polymerase activity

3. Defective induction of the apoptosis pathway

It was decided to focus the work on the three most common mechanisms studied to date looking at the expression of dCK, cN-II and ribonucleotide reductase.

dCK is an essential enzyme in PNA activity as it catalyses the first, but not rate-limiting phosphorylation to the monophosphate (Månsson *et al.* 1999; Gandhi *et al.* 2003). Its loss is frequently implicated in resistance to both purine and pyrimidine NAs both *in vivo* (Kawasaki *et al.* 1993; Kakihara *et al.* 1998; Kroep *et al.* 2002) and *in vitro* (Orr *et al.* 1995; Bai *et al.* 1998; Dumontet *et al.* 1999; Lotfi *et al.* 1999; Månsson *et al.* 1999; Gourdeau *et al.* 2001; Månsson *et al.* 2003).

The role of increased cN-II expression in PNA resistance has been suggested by numerous studies (Carson *et al.* 1991; Kawasaki *et al.* 1993; Schirmer *et al.* 1998; Dumontet et al. 1999; Lotfi *et al.* 2001; Yamamoto *et al.* 2007) and high levels of this enzyme have been associated as a prognostic indicator in acute myeloid leukaemia patients (Galmarini *et al.* 2001; Galmarini *et al.* 2003b)

Increased activity or lack of the inhibitory effects of NAs on ribonucleotide reductase has been reported as a cause of NA resistance. Caras and Martin (1988) showed that mutations in the allosteric regulatory site of RRM1 can cause inhibition of the feedback regulation by dATP. Increased activity of ribonucleotide reductase is thought to cause resistance to NAs due to an increase in dNTPs that can out-compete NAs in other areas such as insertion into DNA or acting as false substrates for DNA polymerases (Parker *et al.* 1991). Several studies have suggested that increased ribonucleotide reductase expression and activity causes resistance to NAs (Carson *et al.* 1992; Dumontet *et al.* 1999; Goan *et al.* 1999; Månsson *et al.* 1999; Davidson *et al.* 2004; Jordheim *et al.* 2005; Ferrandina *et al.* 2010).

The expression of dCK, cN-II and ribonucleotide reductase was investigated at the genomic level by looking at RNA expression, and then at the protein level with Western blotting both to confirm the basal expression and determine clofarabine-induced changes in expression.

5.2 Chapter Aims

The aim of this chapter was:

- To use molecular biological and pharmacological tools to investigate the increased resistance to clofarabine in ER-positive compared to ER-negative breast cancer cell lines.

The specific objectives of this chapter were to:

- Use real-time PCR to investigate RNA levels of genes associated with clofarabine activity including dCK, cN-II and the subunits of ribonucleotide reductase, RRM1, RRM2 and p53R2.
- Use Western blotting to confirm data observed with PCR and to look at differential expression of proteins after treatment with clofarabine.

5.3 Methods

The detailed protocols for the SYBR Green, real-time PCR and Western blotting are described in chapter 2.

5.3.1 RT-PCR

RNA extracted from six breast cancer cell lines (MCF-7, T47D, BT 474, MDAMB231, MDAMB436 and SKBR-3) and HL60 cells was reverse transcribed to cDNA. Using gene-specific primers and a DNA Master SYBR Green I kit, specific cDNA sequences were amplified in a LightCycler 2.0 RT-PCR system (Roche Applied Science, Burgess Hill, UK) under the following conditions:

Program: Denaturation		**Cycles:** 1		
Segment Number	**Target Temperature (°C)**	**Hold Time (sec)**	**Slope (°C/sec)**	**Acquisition Mode**
1	95	600	20	None

Program: Amplification		**Cycles:** 35		
Segment Number	**Target Temperature (°C)**	**Hold Time (sec)**	**Slope (°C/sec)**	**Acquisition Mode**
1	95	10	20	None
2	56	8	20	None
3	72	7	20	Single

Program: Melting Curve		**Cycles:** 1		
Segment Number	**Target Temperature (°C)**	**Hold Time (sec)**	**Slope (°C/sec)**	**Acquisition Mode**
1	95	0	20	None
2	65	15	20	None
3	95	0	0.1	Continuous

Program: Cooling		**Cycles:** 1		
Segment Number	**Target Temperature (°C)**	**Hold Time (sec)**	**Slope (°C/sec)**	**Acquisition Mode**
1	40	30	20	None

The *Ct* values for each gene in each cell line were determined automatically using the second derivative maximum method in the LightCycler v3.5 software (Roche Applied Science, Burgess Hill, UK). All data were normalised to GAPDH expression to control for any differences in the starting amount of RNA. REST software was used to calculate the expression of each gene relative to the expression in the HL60 cells. Therefore, all data are expressed as fold change from the HL60 cells.

Gel electrophoresis was used to confirm amplicon size as described in section 2.6.5 and visualised under UV illumination.

5.3.2 Western blotting

Cell lysates were prepared from cells in logarithmic growth phase and protein quantified as described in sections 2.5.1 and 2.5.3, respectively. To determine the effects of clofarabine cells were treated with the appropriate concentrations for the time period indicated in normal growth media. Media only and vehicle controls were also included. Routinely, 10 µg of protein were separated using SDS-PAGE and proteins were then transferred to nitrocellulose membrane using semi-dry immunoblotting (section 2.5.5). After blocking, membranes were incubated with the primary antibody overnight at 4°C in 1% Blotto before being washed, probed with secondary antibodies, washed again and detected using ECL. The concentrations of primary and secondary antibodies are given below.

β-actin	1°	1:50,000
	2°	1:50,000 anti-mouse
cN-II	1°	1:2000
	2°	1:10,000 anti-rabbit
p53R2	1°	1:2000
	2°	1:10,000 anti-rabbit

Densitometry was used to semi-quantitate protein levels and analysed using ImageJ software (NIH, USA). Data were normalised to β-actin to control for protein loading.

5.3.3 Data Analysis

REST software (REST, Germany) was used to calculate the relative expression of genes compared to the HL60 cells and their significance using a pair-wise fixed reallocation randomisation test from the PCR data. No statistics could be performed to compare the expression between the breast cancer cell lines. This is because the REST software and the relative quantification methods do not allow for statistics to be performed in a cross-wise manner, but only between the genes of interest (GOI) in samples compared to the control (HL60 cells in this instance). See section 2.6.6.

Statistical analyses were carried out in GraphPad Prism V5.0b. Comparisons between two groups were done using a Student's *t* test, and a one-way ANOVA was used for three or more groups with a Tukey-Kramer *post hoc* test if a significant difference was observed between the three or more groups. If only selected means were compared than the Bonferroni correction method was used. Probability values of less than 0.05 were considered statistically significant. Correlations between IC_{50} values and gene expression were determined in GraphPad Prism. The Pearson correlation coefficient (r) was calculated for each gene compared to the IC_{50} value as a Gaussian distribution was assumed but not calculated due to small sample sizes (n=7). Due to the low sample size, a Spearman's Rank test was also used, which doesn't assume normally distributed data and adds confidence to any significant results observed with Pearson's correlation test. The results of the Spearman's Rank test are not included in the results section, but are discussed in the relevant discussion sections of this Chapter.

5.4 Results

5.4.1 RT-PCR validation

SYBR Green, RT-PCR was used to investigate the expression of genes associated with the action of PNA in the six breast cancer cell lines and the HL60 leukaemia cell line. The mRNA expression of dCK, cN-II and the three subunits that form ribonucleotide reductase (RRM1, RRM2 and p53R2) and GAPDH as a control were determined using primers specific for each of these genes.

Figure 5.1 A shows a representative fluorescence trace obtained from HL60 cells looking at the expression of all 5 target genes, and GAPDH as a positive control. Also shown are the minus reverse transcription (-RT) negative controls. Fluorescence signals were seen for all 6 genes indicating their presence. There was no increase in fluorescence above background for the –RT controls. These data were consistent in the other cell lines and repeats of experiments.

Figure 5.1 B shows a representative trace of the melting curve analysis from the same samples as shown in figure 5.1 A. Single peaks were obtained for each of the 6 genes investigated indicating the presence of only one amplicon. The melting points were consistently 3-5 °C higher than expected (see table 2.1, section 2.6.1). There were no peaks present in the –RT control samples. Again, these data were consistently similar with repeats of experiments in all the cell lines.

It was then decided to look at the size of the amplicons to confirm correct amplification of the desired genes. The expected sizes of the amplicons are given in figure 5.2. It can be seen that there are single bands present in each lane that correspond to the expected size of each amplicon.

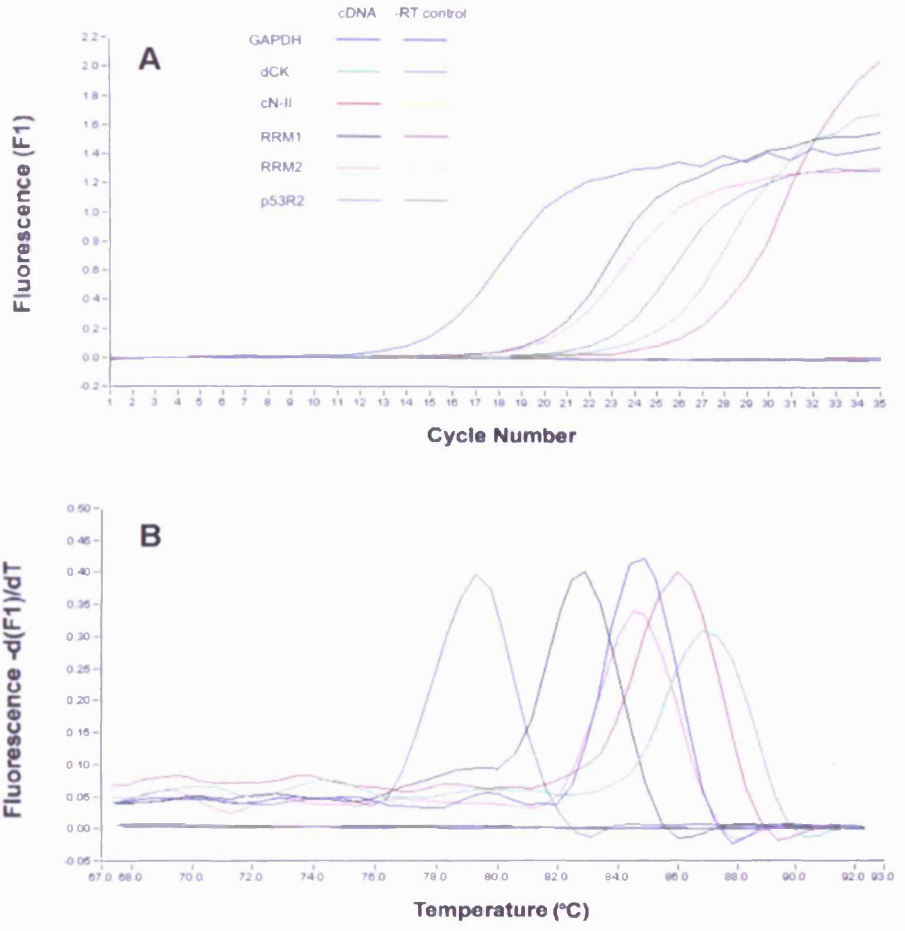


Figure 5.1 Representative Graphs of SYBR Green RT-PCR and Melting Curve Analysis

The top trace (A) is a representative example of HL60 cDNA and subsequent PCR for GAPDH, dCK, cN-II, RRM1, RRM2 and p53R2. The negative controls are also included which were HL60 RNA run under the same conditions but minus the reverse transcriptase enzyme (-RT control). Trace (B) is the melting curve analysis from these samples. Single peaks can be observed in each of the cDNA samples, but no peaks are observed in the –RT controls.

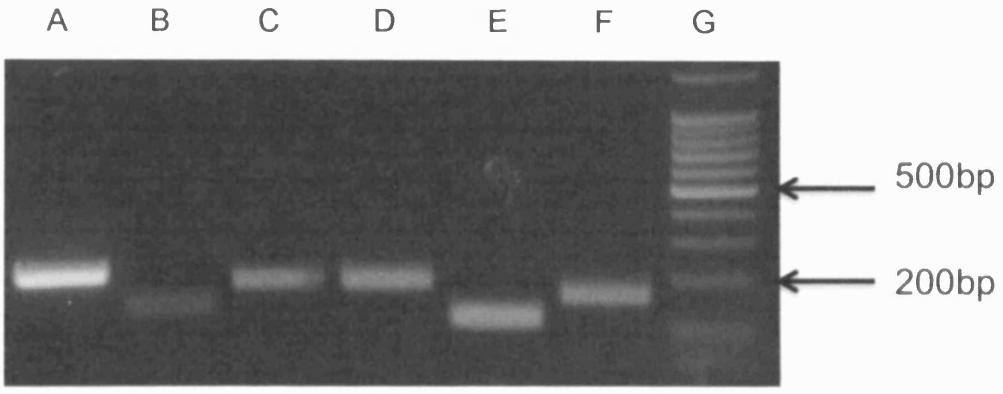


Figure 5.2 Representative Gel of HL60 cDNA RT-PCR Amplicons Separated by Gel Electrophoresis

The image is of HL60 cDNA run on the real-time PCR system without melting curve analysis, and the resulting amplicons were then separated using agarose gel electrophoresis and compared to a ladder of known base pair sizes. A GAPDH (220bp), B dCK (139bp), C cN-II (195bp), D RRM1 (195bp), E RRM2 (120bp), F p53R2 (168bp) and G 100bp Ladder.

5.4.2 Expression of dCK and cN-II

Next, the genomic expression of dCK and cN-II was investigated using the developed real-time PCR method. Figure 5.3 shows the fold expression of dCK and cN-II compared to the HL60 cells for the six different breast cancer cell lines. It can be seen that MCF-7 cells express significantly ($p<0.05$) less dCK RNA than the HL60 cells. There were significant increases ($p<0.05$) in expression of dCK in the T47D and BT 474 cells compared to the HL60 cells.

There were significant ($p<0.05$) increases in cN-II expression in the MCF-7, T47D, BT 474 and MDAMB231 cells compared to HL60 cells (Figure 5.4).

Correlations between gene expression and IC_{50} values of clofarabine were investigated with Pearson's correlation test (r). Figure 5.4 A shows there was no significant ($p>0.05$) correlation with dCK expression and IC_{50} values. Figure 5.3 B, however, shows a significant ($p<0.05$) correlation between cN-II expression and IC_{50} values for clofarabine.

5.4.3 Basal protein expression of cN-II

From the PCR data, it was decided to focus upon the gene that showed positive correlations with the IC_{50} data, cN-II. Firstly, the basal expression of the cN-II protein was determined to see if it correlated with the PCR data. These experiments were carried out in the MCF-7, MDAMB231 and HL60 cells only.

Figure 5.5 A shows a representative example of Western blot probing for cN-II and β-actin as a loading control. Densitometry analysis was performed and showed a significant ($p<0.05$) increase in cN-II protein levels in the MCF-7 cells (0.92±0.07) compared to the MDAMB231 (0.42±0.02) and HL60 (0.18±0.05) cells.

A	Fold RNA Expression Compared to HL 60 Cells (S.E.)	
	dCK	cN-II
MCF-7	0.53 (0.36 - 0.74)#	13.18 (3.52 - 44.53)*
T47D	1.63 (1.27 - 2.28)*	8.54 (2.19 - 29.99)*
BT 474	1.62 (1.46 - 1.84)*	8.96 (2.21 - 31.81)*
MDAMB231	0.78 (0.71 - 0.85)	8.07 (1.99 - 29.52)*
MDAMB436	0.94 (0.60 - 1.37)	4.05 (1.05 - 14.29)
SKBR-3	0.61 (0.43 - 1.06)	2.41 (0.60 - 8.03)

B

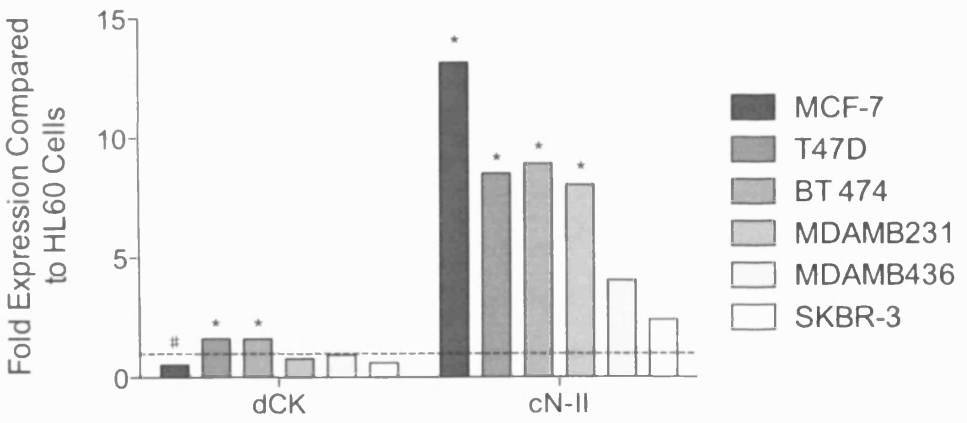


Figure 5.3 Fold Expression of dCK and cN-II RNA in Breast Cancer Cell Lines Compared to HL60 Cells

*This figure (tabulated values A; graphical representation B) shows the fold difference in expression of dCK and cN-II RNA in the six different breast cancer cell lines compared to the HL60 cells assessed by SYBR Green, real-time PCR and REST software. The standard error (S.E.) are shown in brackets (A). The dotted line represents expression in HL60 cells (1). The data are the mean of three separate PCR experiments. *($p<0.05$) upregulated compared to HL60 cells. #($p<0.05$) downregulated compared to HL60 cells. Data were analysed with REST software with a pair-wise fixed reallocation randomisation test.*

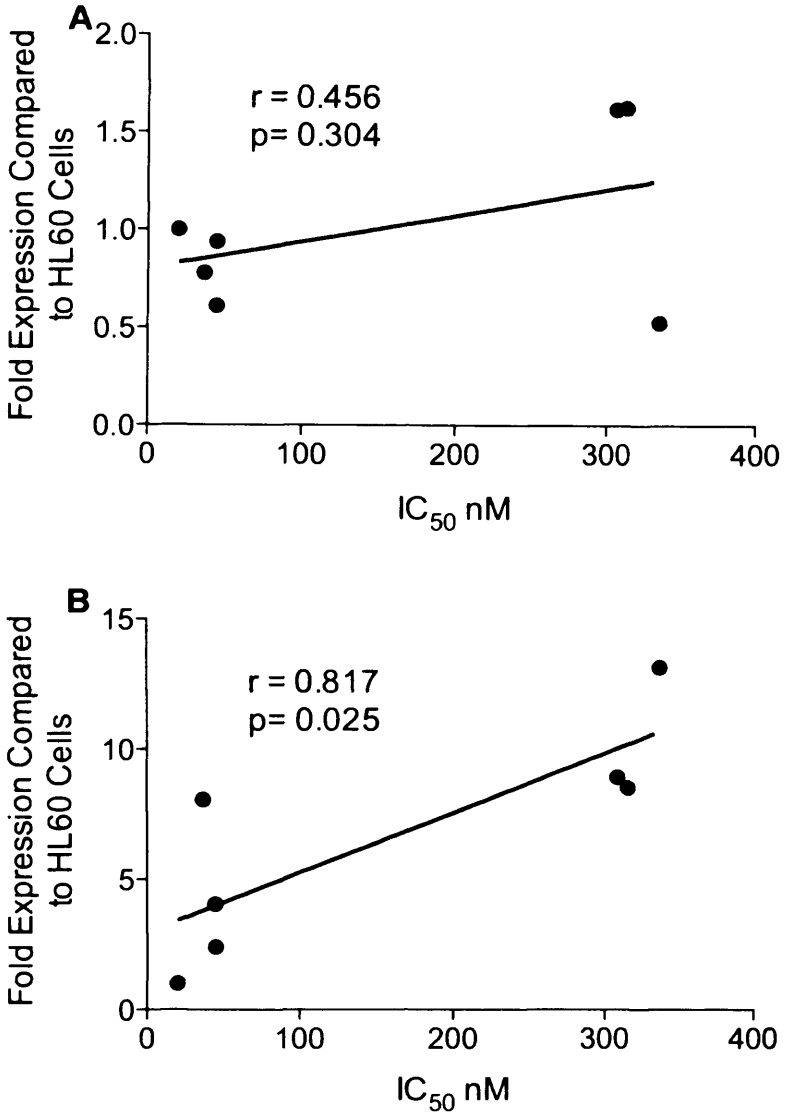


Figure 5.4 Correlation of dCK and cN-II RNA Expression in Breast Cancer Cell Lines with IC_{50} values for Clofarabine

Figures 5.3 A and B show the correlations between the expression of dCK and cN-II, respectively, with the IC_{50} values for clofarabine in the six breast cancer cell lines MCF-7, T47D, BT 474, MDAMB231, MDAMB436 and SKBR-3, and also the HL60 cells. Pearson's correlation coefficient (r) was used to determine the significance of the correlation. $p<0.05$ was considered a statistically significant correlation.

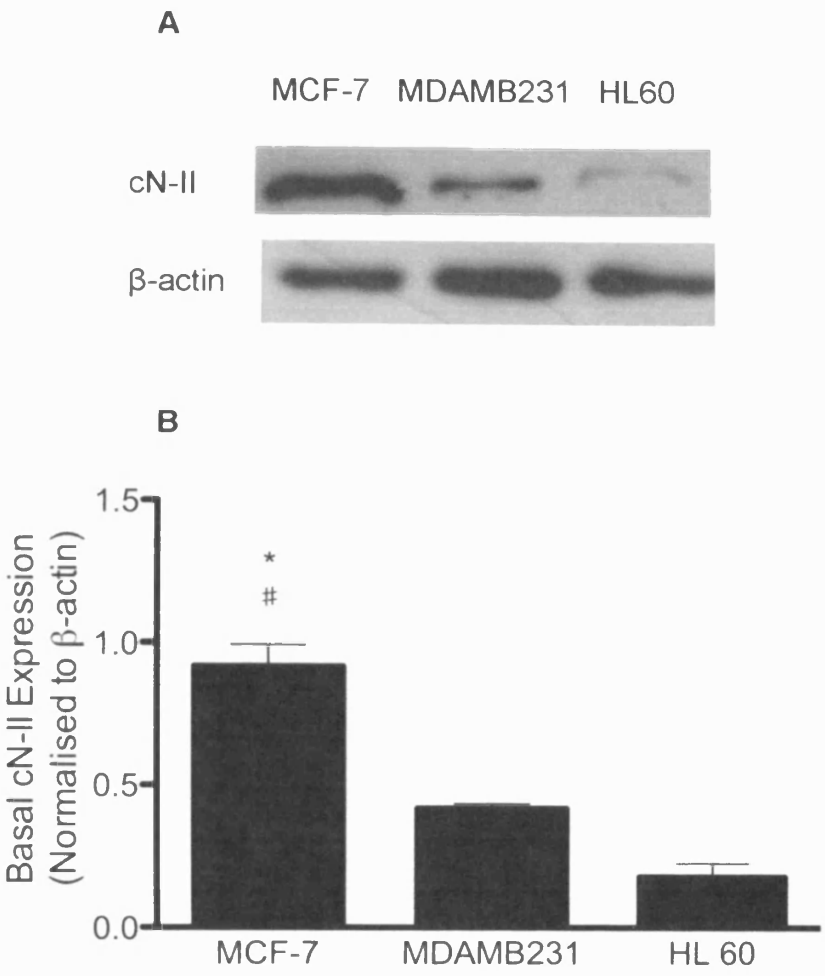


Figure 5.5 Basal Expression of cN-II Protein in MCF-7, MDAMB231 and HL60 Cells

*Cell lysates (10 µg total protein) were subjected to SDS-PAGE electrophoresis, blotted and probed for cN-II and β-actin as a loading control and a representative example is shown (A). Densitometry on scanned images was used to semi-quantitate the levels of cN-II protein, and data were normalised to β-actin (B). Data are expressed as the mean of three separate experiments ± S.E.M. *($p<0.05$) different to HL60 cells. $^{\#}$($p<0.05$) different compared to MDAMB231 cells. Data were analysed with a one-way ANOVA and the Tukey-Kramer post hoc test.*

5.4.4 Expression of cN-II after exposure to clofarabine

It was next decided to investigate whether the protein expression of cN-II was altered after exposure to clofarabine. Using densitometry analysis, no significant ($p>0.05$) differences in expression of cN-II between clofarabine-treated (MCF-7 0.83±0.03 and MDAMB231 0.99±0.10) and control cells were detected. (Figure 5.6).

5.4.5 Expression of ribonucleotide reductase subunits

The RNA expression of the three subunits that make up ribonucleotide reductase was investigated and data are shown in Figure 5.7 A and B.

Fold-expression of the RRM1 subunit was significantly ($p<0.05$) higher in all the cell lines, except for the SKBR-3 cells compared to the HL60 cells. RRM2 gene expression in the BT 474 and MDAMB436 cell lines was significantly higher compared to the HL60 cell lines. MCF-7, T47D and BT 474 cells all had significantly increased ($p<0.05$) p53R2 RNA expression compared to the HL60 control cells. There was a significant decrease ($p<0.05$) in p53R2 protein observed in the SKBR-3 cells compared to the HL60 cells.

Figure 5.8 shows there was a highly significant ($p<0.01$) correlation between p53R2 expression and IC_{50} values for clofarabine, but not for RRM1 or RRM2.

5.4.6 Basal protein expression of p53R2

The expression of basal p53R2 protein was investigated next in MCF-7, T47D, MDAMB231 and HL60 cells. Figure 5.9 A shows a representative blot probed for p53R2 and β-actin.

Figure 5.9 B shows there were approximately equal amounts of p53R2 protein in the MCF-7 (1.41±0.17) and T47D (1.37±0.11) cells which were significantly ($p<0.05$) greater compared to the MDAMB231 (0.62±0.06) and HL60 (0.39±0.03) cells.

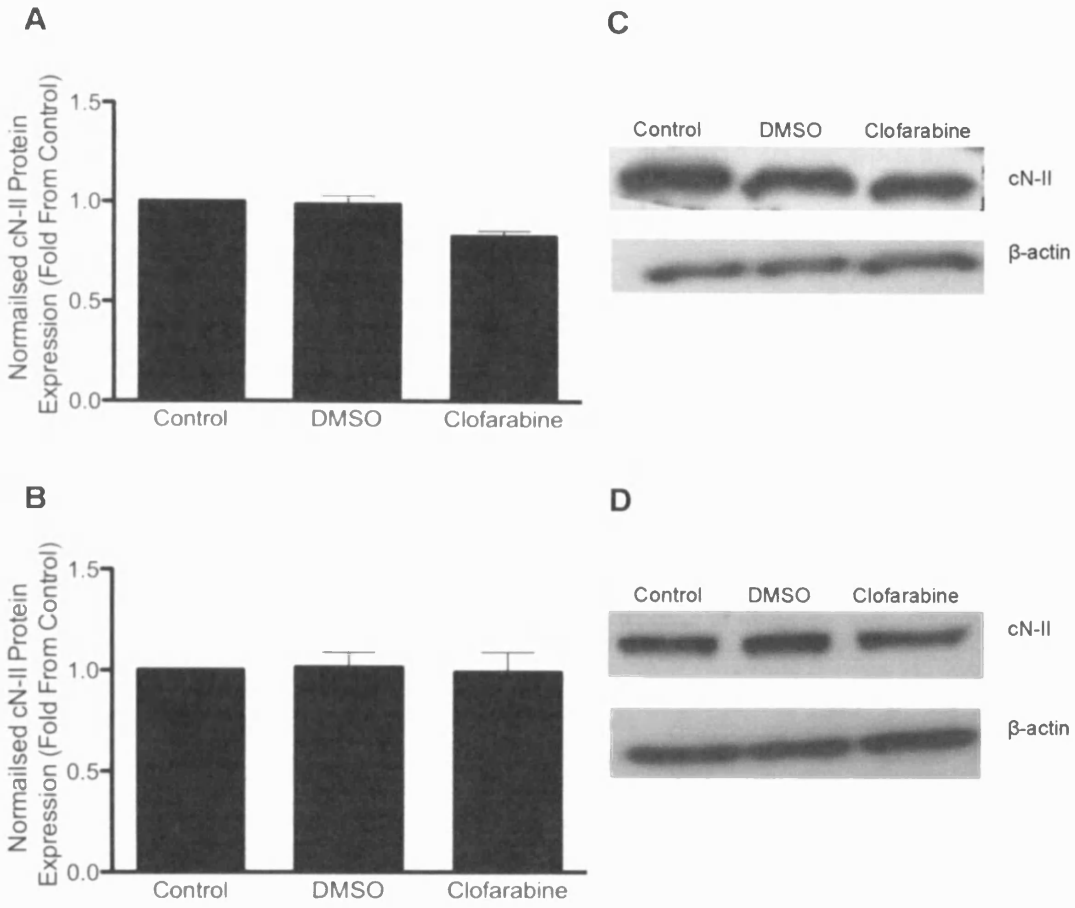


Figure 5.6 Expression of cN-II After Treatment with Clofarabine in MCF-7 and MDAMB231 Cells

This figure shows the expression of cN-II protein after treatment with media only, 0.05% DMSO or 1 μM clofarabine for 96 hours in MCF-7 (A and C) and MDAMB231 (B and D) cells. Representative blots are shown for MCF-7 (C) and MDAMB231 (D) cells. 10 μg total protein was subjected to SDS-PAGE electrophoresis, blotted and probed for cN-II and β-actin. Densitometry on scanned images were used to semi-quantitate the levels of cN-II protein, and data were normalised to β-actin (A and B), and then as a % of control (media only). Data are expressed as the mean of three separate experiments ± S.E.M. No significant ($p>0.05$) differences were observed. Data were analysed with a one-way ANOVA and the Tukey-Kramer post hoc test.

A	Fold mRNA Expression Compared to HL 60 Cells (S.E.)		
	RRM1	RRM2	p53R2
MCF-7	1.42 (1.29 - 1.65)*	1.16 (0.97 - 1.32)	10.08 (5.10 - 17.83)*
T47D	3.00 (2.82 - 3.20)*	2.43 (2.00 - 2.88)	7.60 (6.06 - 9.10)*
BT 474	2.00 (1.87 - 2.22)*	2.51 (2.19 - 2.97)*	4.45 (3.98 - 4.95)*
MDAMB231	2.53 (1.71 - 3.20)*	1.18 (0.99 - 1.43)	1.69 (0.99 - 2.51)
MDAMB436	2.74 (1.23 - 3.18)*	4.94 (4.38 - 5.84)*	2.31 (1.56 - 3.45)
SKBR-3	0.73 (0.61 - 0.88)	1.29 (1.06 - 1.50)	0.40 (0.38 - 0.42)#

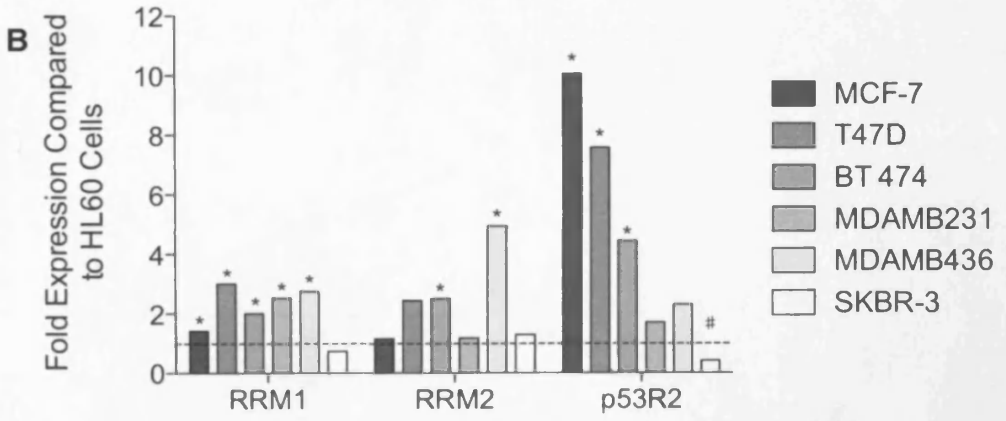


Figure 5.7 Fold Expression of RRM1, RRM2 and p53R2 RNA in Breast Cancer Cell Lines Compared to HL60 Cells

*This figure (tabulated values A; graphical representation B) shows the fold difference in expression of RRM1, RRM2 and p53R2 RNA in the six different breast cancer cell lines compared to the HL60 cells assessed by SYBR Green, real-time PCR and REST software. The standard errors (S.E.) are shown in brackets (A). The data are the mean of three separate PCR experiments. Data were analysed with REST software with a pair-wise fixed reallocation randomisation test The dotted line represents expression in HL60 cells (1). *($p<0.05$) upregulated compared to HL60 cells. #($p<0.05$) downregulated compared to HL60 cells.*

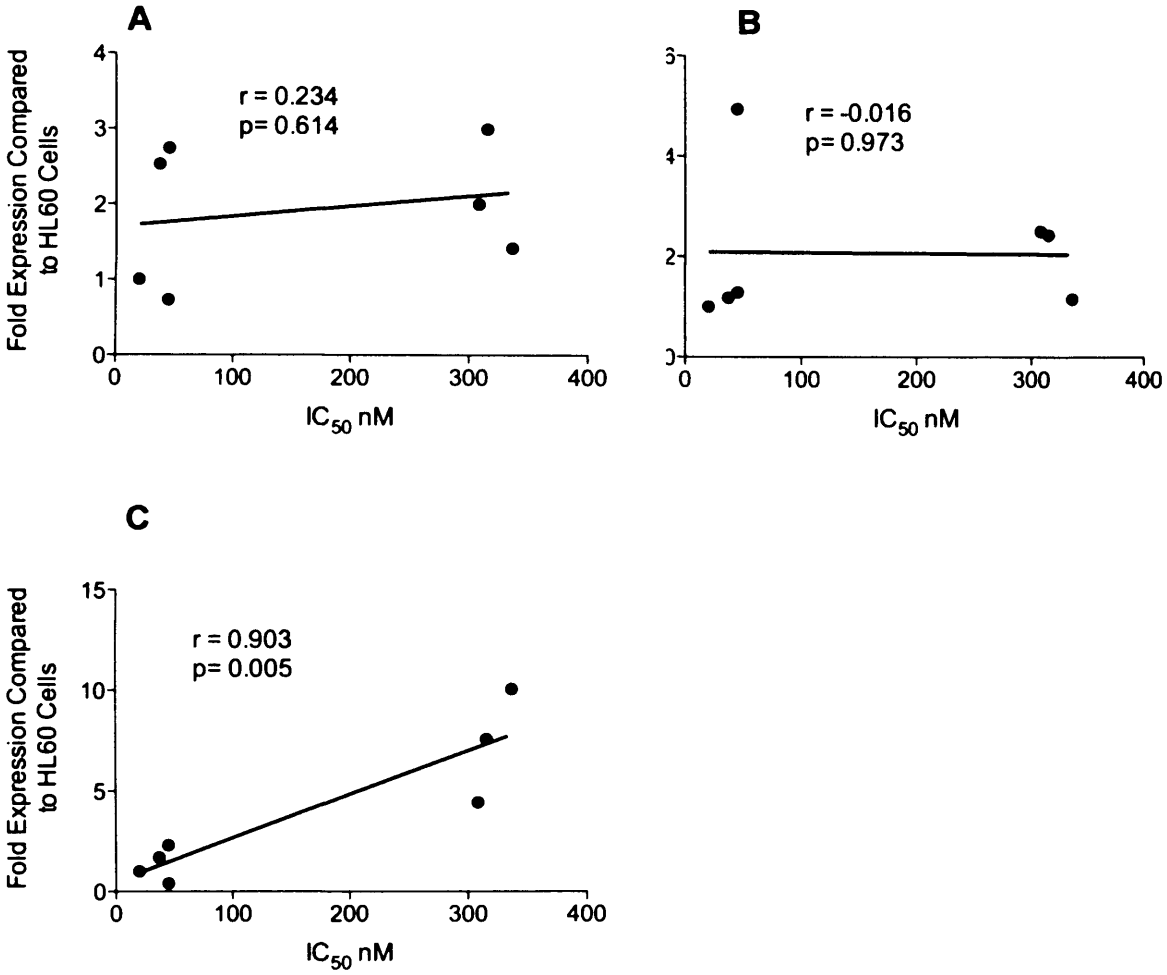


Figure 5.8 Correlation of RRM1, RRM2 and p53R2 RNA Expression in Breast Cancer Cell Lines with IC_{50} value for Clofarabine

A, B and C show the correlations between the expression of RRM1, RRM2 and p53R2, respectively, with the IC_{50} values for clofarabine in the six breast cancer cell lines MCF-7, T47D, BT 474, MDAMB231, MDAMB436 and SKBR-3, and also the HL60 cells. Pearson's correlation coefficient (r) was used to determine the significance of the correlation. $p<0.05$ was considered a statistically significant correlation.

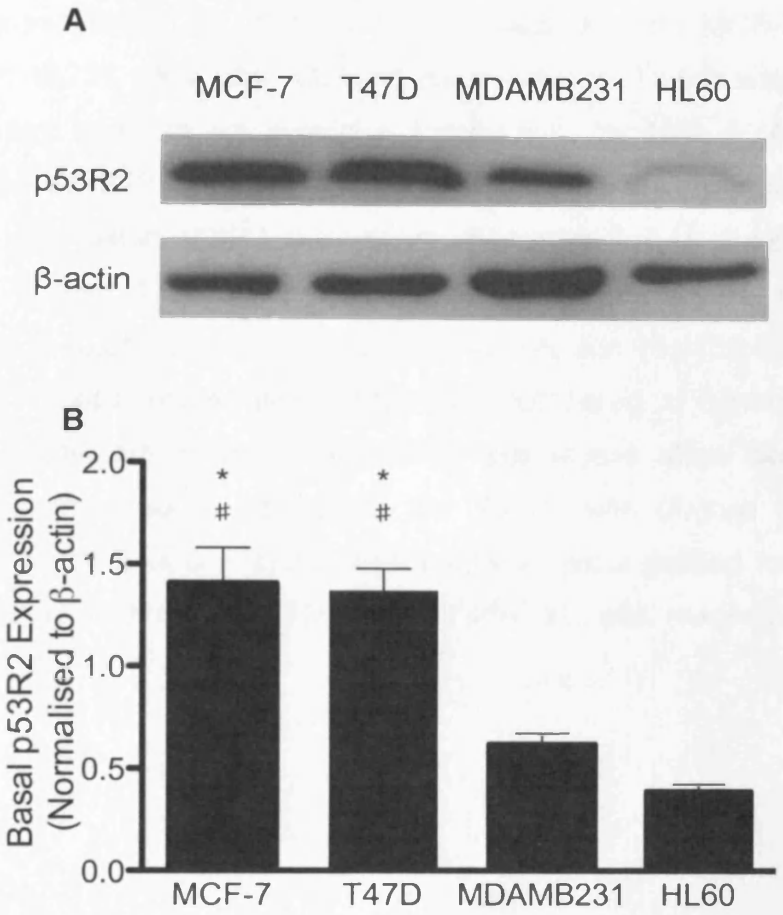


Figure 5.9 Basal Expression of p53R2 Protein in MCF-7, T47D, MDAMB231 and HL60 Cells

*Total cell lysates (10 μg protein) were subjected to SDS-PAGE electrophoresis, blotted and probed for p53R2 and β-actin as a loading control and a representative example is shown (A). Densitometry on scanned images was used to semi-quantitate the levels of p53R2 protein, and data were normalised to β-actin (B). Data are expressed as the mean of three or four separate experiments ± S.E.M. *($p<0.05$) different to HL60 cells. #($p<0.05$) different compared to MDAMB231 cells. Data were analysed with a one-way ANOVA and the Tukey-Kramer post hoc test.*

5.4.7 Expression of p53R2 after exposure to clofarabine

Next, the expression of p53R2 was investigated in the MCF-7, T47D and MDAMB231 cells after clofarabine treatment. The results of the densitometry analysis are shown in Figure 5.10 for MCF-7 (A), T47D (B) and MDAMB231 (C). It can be seen that there was a significant ($p<0.05$) increase in p53R2 expression upon exposure to clofarabine in the MCF-7 cells (1.62±0.11) (Figure 5.10 A). Figure 5.10 C shows a significant ($p<0.05$) decrease in p53R2 expression (0.61±0.06) in the MDAMB231 cells treated with clofarabine compared to control. There was no significant change in p53R2 expression after clofarabine treatment compared to control in the T47D cells (Figure 5.10 B). Figures 5.10 D, E and F show representative blots probed for p53R2 and β-actin in the MCF-7, T47D and MDAMB231 cells, respectively.

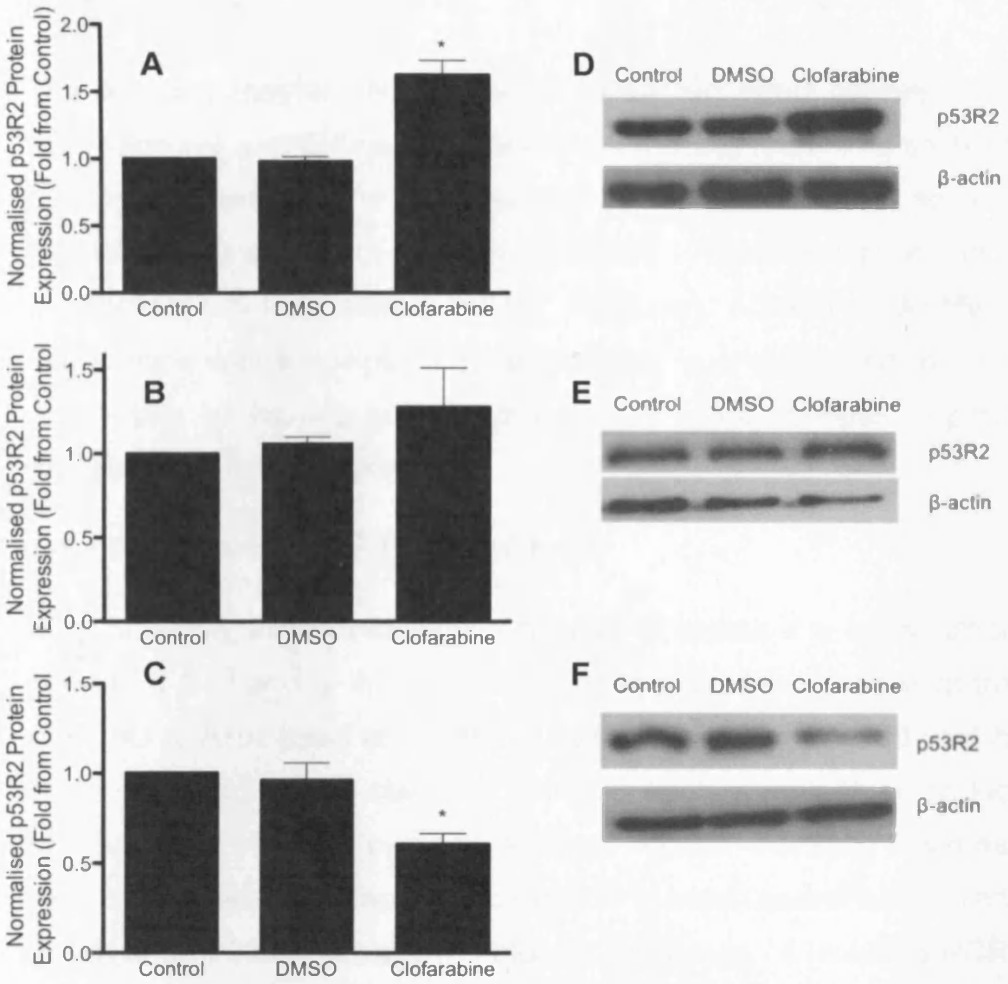


Figure 5.10 Expression of p53R2 After Treatment with Clofarabine in MCF-7, T47D and MDAMB231 Cells

*This figure shows the expression of p53R2 protein after treatment with media only, 0.05% DMSO or 1 μM clofarabine for 96 hours in MCF-7 (A and D), T47D (B and E) and MDAMB231 (C and F) cells. Representative blots are shown for MCF-7 (D), T47D (E) and MDAMB231 (F) cells. 10 μg total protein was subjected to SDS-PAGE electrophoresis, blotted and probed for p53R2 and β-actin. Densitometry on scanned images were used to semi-quantitate the levels of p53R2 protein, and data were normalised to β-actin (A, B and C), and then as a % of control (media only). Data are expressed as the mean of three separate experiments ± S.E.M. *($p<0.05$) different control. Data were analysed with a one-way ANOVA and the Tukey-Kramer post hoc test.*

5.5 Discussion

The previous chapter had identified a differential effect between PNAs in ER-positive and ER-negative breast cancer cell lines. The six breast cancer cell lines and the HL60 leukaemia cell line were first screened for RNA expression of 5 GOI related to PNA activation (dCK), metabolism (cN-II) or activity (RRM1, RRM2 and p53R2) to identify any correlations with IC_{50} data. Western blotting was then used to further these data by looking at basal and drug-induced changes in protein expression of relevant genes.

5.5.1 Confirmation of RT-PCR method

PCR is widely used in molecular biology to assess the transcriptional state of a GOI and is the principal technique available to look at trace amounts of RNA (Heid *et al.* 1996). Real-time PCR was used as it has many advantages over standard 'semi—quantitative' PCR which looks at the end product by gel electrophoresis, including increased sensitivity, specificity, reproducibility and a wider quantification range (Pffafl *et al.* 2006). Perhaps the biggest advantage of real-time PCR is that the quantification of the amplification product occurs during exponential amplification, not as an end-point as with traditional 'semi—quantitative' PCR using gel-electrophoresis and densitometry analysis. (Lockey *et al.* 1998).

Quantitative real-time PCR depends upon comparing RNA copy numbers to a calibration curve and results provide an 'absolute' quantification. However, the standards for the GOI looked at in this study could not be acquired, therefore, a relative quantification system was used. This involves looking at differences in RNA expression from different samples or cell lines and comparing them to a control sample. In this instance, the HL60 cell line was chosen as the control sample and all data are expressed as fold-change from this. The data are also normalised to a constantly expressed housekeeping gene, in this

instance, GAPDH, to control for any differences in starting quantity of RNA/cDNA and inter-experimental variability. As this relative quantification method can be used to compare data across multiple real-time PCR experiments and is used to investigate small changes in gene expression across samples (Pffafl *et al.* 2006) it was therefore used for these studies.

Figure 5.1 A shows a representative fluorescent trace of the five GOI and GAPDH from the HL60 cell line. It can be seen that increases in fluorescence are obtained for all 6 genes, indicating the HL60 cells were transcribing RNA from all six genes. GAPDH RNA was the most abundant, and cN-II RNA was the most sparse.

To ensure that only RNA had been extracted from the cells and not DNA, controls were included in each experiment. These controls were treated the same as the samples, except during the reverse-transcription step, the reverse transcriptase enzyme was omitted which means that if only RNA was extracted, no cDNA would be produced. Therefore, during the PCR there should be no amplification as the primers will only bind to and amplify from DNA. These controls also served to confirm there were no contaminants in any of the reagents. It can be seen that there was no increase in fluorescence suggesting that no amplification took place and there was no contaminating DNA. This was done for all the samples in all the cell lines to confirm integrity. Subsequent PCR runs were completed with H_20 controls where the cDNA was replaced with H_20 to ensure that each PCR run was free from contaminants.

To confirm specific amplification of the correct sequence of DNA, melting curve analysis can be used with real-time PCR. This involves steadily heating the samples at the end of the amplification stage progressively to 95°C and determining the temperature at which the fluorescence signal rapidly deteriorates. This melting temperature (T_m) of an amplicon is the temperature at which the DNA strands separate

from one another and is determined by factors such as base pair composition, amplicon length and PCR conditions. The presence of a single peak in the melting curve analysis indicates only one amplicon is present, and the temperature at which this occurs helps to define what this amplicon is. Figure 5.1 B, shows the melting curve analysis from Figure 5.1 A. Single peaks are observed for each of the six GOI, indicating only one amplicon was present. No peaks were observed for the controls meaning that no amplicons were produced, confirming the data in Figure 5.1 A. However, the T_m observed were all 3-5 °C higher than the calculated T_m (see Table 2.1, section 2.6.1). This could be due to slight increases in potassium or sodium salts that caused an increase in the temperature needed to denature the double-stranded DNA. To confirm that these amplicons were in fact specific to the GOI, the PCR was rerun minus the melting curve analysis and the PCR products were separated and sized using gel electrophoresis. The sizes of the bands were compared to a 100bp ladder. The amplicons for the six genes were all at the correct size; GAPDH 200bp, dCK 139bp, cN-II 195bp, RRM1 195bp, RRM2 120bp and p53R2 168bp. This confirmed that the fluorescence signals generated during the PCR were specific to the GOI.

5.5.2 The expression of cN-II, but not dCK is correlated with the potency of clofarabine

The expression of dCK and cN-II was investigated first as these are the two principle enzymes involved in PNA activation and metabolism (Bonate *et al.* 2006). There was a significant up-regulation of dCK gene expression in the T47D and BT 474 cell lines compared to the HL60 cells, whereas the other breast cancer cell lines all had decreased dCK gene expression in comparison. Previous studies have identified that cells of lymphoid or myeloid origin have highest basal levels of dCK enzyme activity, compared to normal tissue or other cancers including head and neck, ovarian, pancreatic, lung and colon cancers. It is also

known that solid tumours express higher levels of dCK compared to their normal, non-cancerous tissues (Eriksson *et al.* 1994; Spasokoukotskaja *et al.* 1995; van der Wilt *et al.* 2003). The data here suggest that some phenotypes of breast cancer may have increased levels of dCK expression compared with leukaemic cells. Confirming these data with Western blotting to look at the actual protein was attempted, however, the antibody used was poor and even at dilutions of 1:25 probing 50 µg of protein, no specific signal was found. As there was no correlation between dCK expression and the IC_{50} values for clofarabine, it was decided not to pursue this further. A Spearman's Rank test confirmed no correlation between gene expression and IC_{50} value ($r=-0.04$; $p=0.96$).

Although a significant number of studies have suggested a role for dCK in determining drug activity (Galmarini *et al.* 2003b) or resistance to PNA (Orr *et al.* 1995; Bai *et al.* 1998; Dumontet *et al.* 1999; Lotfi *et al.* 1999; Månsson *et al.* 1999; Gourdeau *et al.* 2001; Månsson *et al.* 2003), the data here do not suggest this. Whilst dCK is clearly an important enzyme as shown in the previous chapter (see section 4.4.7), its genomic expression does not correlate with the potency of clofarabine. Correlations were not determined with fludarabine or cladribine as the effects of these drugs were only studied in two breast cancer cell lines. Similarly, these data are also supported by the protide data in the previous chapter. It was concluded that by-passing the primary phosphorylation by dCK did not improve the potency or efficacy of cladribine or clofarabine, suggesting that a) dCK is not rate-limiting, and b) dCK expression does not correlate with drug potency.

Studies by Stegmann *et al.* (1995) and Hapke *et al.* (1996) found that retroviral transfer of dCK genes into deficient cell lines, including MCF-7, increased sensitivity to cladribine, cytarabine and fludarabine. This suggests that although PNA resistance is probably determined mostly by other factors, the level of dCK may still contribute to PNA potency. It

is possible that past a certain threshold, the level of dCK is no longer a deciding factor in PNA potency, which is supported by the fact that dCK is not rate-limiting (Gandhi *et al.* 2003).

As pyrimidine analogues activity are rate-limited by dCK, these data may explain the equal potency of gemcitabine in the previous chapter as there was little difference in the expression of dCK between MCF-7 and MDAMB231 cells (0.25 –fold).

The level of the dephosphorylating enzyme, cN-II was investigated next. There are limited data on the expression of cN-II between normal and neoplastic tissues, and between cancer types. However, cN-II is generally thought to be up-regulated in cancer cells compared to normal tissue (Vannoni *et al.* 2004; Suzuki *et al.* 2007), although studies by Rosi *et al.* (1998a; 1998b) found the opposite to be true.

Interestingly, there was a significant positive correlation observed ($r=0.82$) between increased levels of cN-II RNA and decreased potency of clofarabine. Similarly, a Spearman's Rank test also produced a significant correlation, further confirming these data ($r=0.85$; $p=0.02$). In order to confirm these data, the expression of the cN-II protein was determined in the MCF-7, MDAMB231 and HL60 cell lines. Indeed these data are similar to those observed with the PCR. However, the difference in cN-II protein levels between cell lines was not the same as the difference between RNA expression, suggesting that post-translational modifications may contribute to cN-II activity and regulation. These data do, however, confirm further the role of basal cN-II in increased resistance to clofarabine and PNA.

This idea has been suggested widely in the literature (Carson *et al.* 1991; Kawasaki *et al.* 1993; Schirmer *et al.* 1998; Dumontet *et al.* 1999; Lotfi *et al.* 2001; Yamamoto *et al.* 2007). It has also been used as a prognostic indicator to activity of PNA analogues (Galmarini *et al.* 2001; Galmarini *et al.* 2003b) and gemcitabine (Sève *et al.* 2005; Qu *et al.*

2007). Studies by Giovannetti *et al.* (2005) found that inhibition of cN-II with diethylpyrocarbonate could increase the activity of gemcitabine in human lung and epidermoid carcinoma cell lines. These data may suggest a role for cN-II as a prognostic indicator in determining PNA potency in breast cancer, should these drugs make it into the clinic as breast cancer therapeutics. Indeed, several studies have found that cN-II expression correlates with response to PNA in the clinic (Kawasaki *et al.* 1993; Galmarini *et al.* 2001; Galmarini *et al.* 2003b).

Determining the enzyme activity would also be useful to ensure that enzyme levels correlate with actual activity. Indeed, numerous studies have used this approach with dCK and cN-II and found similar results between expression and activity (Kawasaki *et al.* 1993; Kawasaki *et al.* 1996; Månsson *et al.* 1999; Månsson *et al.* 2003). However, a study by Lotfi *et al.* (2006) found that there was no correlation between mRNA expression of dCK and dCK activity in isolated leukocytes from patients with B-cell leukaemia, suggesting post-translational modifications are central to dCK activity.

Also, determining the exact role of cN-II in PNA resistance would be of use. Although it is widely assumed that overexpression of metabolising enzymes is related to direct metabolism of PNA, other studies have found that cN-II overexpression modulates dNTP pools causing resistance. Cytarabine resistance has been associated with cN-II overexpression, but it is not a substrate for cN-II (Mazzon *et al.* 2003). However, Albertioni *et al.* (2005) found that degradation of fludarabine monophosphate is correlated with cN-II activity, supporting the idea that cN-II is acting via direct deactivation of PNA. This study also found a correlation between cladribine monophosphate degradation and cN-I activity, suggesting that activity of this nucleotidase is also involved in PNA deactivation.

cN-II is allosterically activated by purine nucleotides, with ATP being the strongest activator, followed by dATP, but is also dependent upon the

concentration of organic phosphate which acts to deactivate the enzyme (Walldén *et al.* 2007). It was decided to see if clofarabine could alter the expression of the cN-II protein in the MCF-7 or MDAMB231 cell lines. Ideally, one would look at the activity of the enzyme after treatment with clofarabine using enzyme kinetics studies. Due to time constraints these studies were not completed. Figure 5.6 shows that clofarabine (1 μM) had no effect on the expression of cN-II in either cell line. This suggests that the role of cN-II in resistance to clofarabine is dependent upon the basal expression of cN-II enzyme, not by drug-induced changes. These data do not, however, suggest whether cN-II is more or less active in response to clofarabine exposure and only limited conclusions can be made.

5.5.3 The expression of p53R2, but not RRM1 or RRM2 is correlated with the potency of clofarabine

Cladribine and clofarabine were both found to be more potent compared to fludarabine in the MCF-7 and MDAMB231 cell lines (chapter 4). dATP is an inhibitory allosteric effector and, by binding to the allosteric site on RRM1, inhibits the activity of ribonucleotide reductase (Jordan and Reichard 1998). PNA triphosphates have increased affinity for the RRM1 allosteric binding site compared to dATP and are thus powerful inhibitors of ribonucleotide reductase (Shao *et al.* 2006). As cladribine and clofarabine are 10-fold more potent inhibitors of ribonucleotide reductase than fludarabine (Parker *et al.* 1991) this suggests that inhibition of this enzyme is more critical to their mechanisms of action and also suggests that intracellular levels of this enzyme also help dictate sensitivity to NAs. The data from the previous chapter also highlighted a potential role of ribonucleotide reductase in determining potency of clofarabine and so the basal RNA expression of the three subunits of ribonucleotide reductase, RRM1, RRM2 and p53R2 was investigated next.

The RRM1 subunit was significantly up-regulated in the MCF-7, T47D, BT 474, MDAMB231 and MDAMB436 cell lines compared to the HL60 cells. There was no correlation between RNA expression and IC_{50} values for clofarabine (r=0.23) suggesting that RRM1 expression is not a determining factor in clofarabine potency. A Spearman's Rank also produced no significant correlation (r=0.27; p=0.56). Significant links between gemcitabine potency and RRM1 levels have been suggested previously where increased RRM1 levels correlated with increased resistance to gemcitabine in leukaemic, breast and pancreatic cancer cell lines (Davidson *et al.* 2004; Jordheim *et al.* 2005; Nakahira *et al.* 2007). Månsson *et al.* (2003) found that in CEM leukaemia cells resistant to clofarabine, cladribine and fludarabine, there was no change in expression of the RRM1 protein suggesting it is not involved in resistance. This highlights the potential differences in resistance mechanisms to different types of analogue, either purine or pyrimidine.

The RNA expression of RRM2 was significantly increased in the BT 474 and MDAMB436 cell lines only. Again there was no correlation between expression of RRM2 and IC_{50} values for clofarabine (r=-0.02) suggesting that RRM2 expression is not a determining factor in clofarabine potency either. This was again confirmed with a Spearman's Rank test (r=0.27; p=0.56). Studies by Månsson *et al.* (1999; 2003) found that fludarabine-resistant CEM cells had increased RRM2 subunit expression over their wild-type parental cell line, suggesting overexpression of RRM2 in fludarabine resistance. With the use of a ribonucleotide reductase inhibitor, didox, in combination with fludarabine, sensitivity to fludarabine in the fludarabine-resistant CEM cells was restored. This suggested that increased ribonucleotide reductase activity was contributing to fludarabine resistance. This has also been demonstrated in cells resistant to gemcitabine (Duxbury *et al.* 2004; Giovannetti *et al.* 2005; Nakano *et al.* 2007). What these data highlight is the specific nature of NAs and their resistance mechanisms, where cells resistant to a particular NA are not necessarily resistant to

another NA by the same mechanism, particularly true for fludarabine and the two other analogues, cladribine and clofarabine. As such, resistance to NA should be approached in a cell and drug-specific manner.

When the expression of p53R2 was investigated, there was a significant up-regulation of this gene in the ER-positive breast cancer cells between 10- and 4-fold over the HL60 cells. Whereas the SKBR-3 cells had a 0.6-fold decrease in expression. When the genomic expression of p53R2 was correlated with IC_{50} values for clofarabine there was a highly significant positive correlation ($r=0.90$, $p=0.005$) showing increased expression correlates with decreased sensitivity. Nonparametric testing of this correlation with a Spearman's Rank test further validated these data and found a significant correaltion ($r=0.85$; $p=0.02$). To confirm these data, the expression of the p53R2 protein was investigated in the MCF-7, T47D, MDAMB231 and HL60 cells. There were approximately equal amounts of p53R2 protein in the MCF-7 and T47D cells , 1.41 and 1.37 fold, respectively. The MDAMB231 cells had less than 50% of this amount (0.62), and the HL60 cells even less (0.39). These data agree with the PCR data, although there is a difference in the ratio of protein expression between the cell lines compared with the PCR data, suggesting post-translational regulation of this enzyme subunit.

The basal expression of p53R2 could be linked to its role in supplying dNTPs to mitochondrial DNA synthesis as a study by Bourdon *et al.* (2007) found that p53R2 gene mutations and reduced function resulted in depletion of mitochondrial DNA. This theory is also suggested by Pontarin *et al.* (2007). It is known that p53R2 is constitutively expressed in cells regardless of their p53 status, p53 status is important only in its overexpression after DNA insult (Wang *et al.* 2009).

Previous studies have indentified a correlation between increased p53R2 expression and reduced invasion and metastatic potential in

colon carcinoma tissue, and suppression of p53R2 increased invasion and migration (Liu *et al.* 2006; Liu *et al.* 2007). These data are in agreement with the results here where ER-negative tumours, which are more aggressive and show increased invasiveness, express significantly less p53R2 than ER-positive tumours. Okumura *et al.* (2006), however, found that in patients with oesophageal squamous cell carcinoma, positive expression of p53R2 correlated significantly with increased tumour invasion, lymph node involvement and high grading of tumours compared with those deemed p53R2-negative. The data in the study here do not follow this pattern as ER-negative tumours are associated with tumours that have a histologically high grading (Rochefort *et al.* 2003). These data suggest that p53R2 expression and related cell grading or invasiveness is cell and/or cancer type-dependent and suggests that inferring data from one cancer type to another should be done with caution.

The expression of p53R2 was investigated after treatment with clofarabine for 96 hours. This expression was also investigated in the T47D cells as these cells are p53-mutated, but ER-positive and this would determine if p53R2 expression is p53-dependent. There was a 60% increase in p53R2 expression over control following treatment with clofarabine in the MCF-7 cells. Although there was a 28% increase in p53R2 expression in the T47D cells compared to control, this was not significant. Interestingly, there was a significant 40% reduction in p53R2 expression following treatment with clofarabine in the MDAMB231 cells. The reasons for this decrease in p53R2 expression are unclear and not readily explainable, but this decrease in p53R2 may explain the increase in sensitivity to clofarabine in these cells. One possibility is that clofarabine is disrupting the gene locus of p53R2 (chromosome 8q23.1; Smeds *et al.* 2001) causing mutation, DNA damage and ultimately affecting transcription. Månsson *et al.* (2003) found that clofarabine-resistant CEM cells showed increased structurally rearranged

chromosomes suggesting that clofarabine is able to disrupt the genome more efficiently than other PNAs.

These data indicate that in p53 wild-type cells (MCF-7), clofarabine can induce p53R2 expression but this expression cannot be induced in p53 mutant cells, regardless of ER-status or basal expression (T47D and MDAMB231). These findings are consistent with the literature demonstrating an increase in p53R2 protein expression following DNA damage by cytotoxics or UV irradiation (Nakano *et al.* 2000; Tanaka *et al.* 2000; Barber *et al.* 2005; Tsai *et al.* 2006; Link *et al.* 2008).

Lin *et al.* (2007) observed an interesting response to DNA damaging agents in HC116 colon cancer cells that are p53-null. Their study found that the RRM2 subunit could effectively take the place of p53R2 and provide dNTP for DNA repair after treatment with cisplatin. This idea has been supported by Zhang *et al.* (2009) who found that camptothecin treatment up-regulated RRM2 expression and silencing of RRM2 enhanced DNA damage caused by camptothecin in MDAMB231 cells. This may mean that that MDAMB231 cells are able to repair their DNA by RRM2-mediated mechanisms. If this is the case with clofarabine-induced DNA damage, then this suggests that the differential response to clofarabine is not solely mediated by DNA repair mechanisms, or lack thereof. Interestingly, the MDAMB436 cells showed over 4-fold higher expression of RRM2 compared to the MDAMB231 cells, yet they were equally susceptible to clofarabine, and neither the MDAMB231 nor the MDAMB436 cells should be able to induce p53R2 in response to DNA damage due to mutated or lack of p53, respectively. These data suggest that RRM2-mediated DNA repair does not affect clofarabine potency. This may be due to clofarabine-induced DNA damage not activating the repair signal initiated by checkpoint kinase 1 for RRM2-mediated DNA repair (Zhang *et al.* 2009) or that RRM2-mediated repair is drug-specific.

As p53R2 expression is increased in response to DNA damage, and is involved in DNA repair, these data imply that the resistance to clofarabine is caused by DNA repair of the genomic insult. The fact that only the MCF-7 cells had an increase in p53R2 expression after clofarabine treatment further suggests that p53R2 is p53-dependent (Tanaka *et al.* 2000), and breast cancer cells with mutated p53 i.e. T47D and MDAMB231, are unable to initiate this response. This suggestion of p53R2-mediated resistance is supported by another factor. In the previous chapter, gemcitabine was identified as equally potent in both MCF-7 and MDAMB231 cells, suggesting a mechanism unique to gemcitabine that clofarabine does not induce/target. Wang *et al.* (2009b), found that gemcitabine diphosphate was able to inhibit p53R2, something not yet identified with clofarabine.

These studies have only touched the surface as to the role of p53R2 in determining sensitivity to PNA in human breast cancer cell lines and much work remains to investigate this relationship further. To confirm the role of either basal or inducible p53R2 in sensitivity to PNA, gene silencing or knockout studies would provide the most reliable answer to this. siRNA techniques have been used in the literature and show that knockout of p53R2 increases cancer cells' susceptibility to cytotoxic agents and radiation treatments *in vitro* (Yanamoto *et al.* 2005; Devlin *et al.* 2008). Similarly, using more specific pharmacological inhibitors of ribonucleotide reductase than hydroxyurea (chapter 4) such as triapine would help clarify the role of ribonucleotide reductase as a whole in the mechanisms of action and resistance to clofarabine. Looking at basal and clofarabine-induced protein expression of p53R2 in the BT 474, MDAMB436 and SKBR-3 cells would also lend more weight to some of the arguments within this thesis.

Again using siRNA techniques, the role of cN-II in determining resistance to clofarabine could be elucidated. If high expression of cN-II is mediating, at least in part, the sensitivity of breast cancer cells to

clofarabine, then its reduction should increase this sensitivity. It may also be useful to determine more accurately the quantification of the expression of dCK and cN-II so that a ratio between them can be investigated. Real-time PCR could be used again, but using absolute rather than relative quantification methods utilising standards of dCK and cN-II. Better antibodies against dCK could also be used to allow semi-quantitative measurements of this enzyme to be made.

5.6 Conclusion

In this chapter the expression of enzymes related to the activity of clofarabine was investigated and related to the differential effects of clofarabine in ER-positive and ER-negative breast cancer cell lines.

There appears to be no correlation between the expression of the activating enzyme dCK and the potency of clofarabine. However, there was a positive correlation between the expression of cN-II RNA and the differential potency of clofarabine and this was confirmed at the protein level in three cell lines also. Increased breakdown of clofarabine nucleotides by cN-II may account for some of the reduced effects of clofarabine in the ER-positive breast cancer cell lines, compared to the ER-negative breast cancer cells and HL60 leukaemia cells.

There was a strong correlation between clofarabine potency and the expression of the p53-inducible R2 subunit, p53R2 and this was again confirmed at the protein level. Similarly, there was an increase in p53R2 protein expression following exposure to clofarabine in the p53 wild-type MCF-7 cells, but not in two p53-mutant cell lines regardless of ER status (T47D and MDAMB231). These data suggest that p53R2 plays a role in determining sensitivity to clofarabine in breast cancer cell lines. This is probably due to increased DNA repair capabilities in cell lines expressing higher levels of p53R2.

These data need confirmation by further experimentation and are unlikely to be the only explanation for the resistance to clofarabine in ER-positive cells lines. There are probably multiple factors that contribute to the varying sensitivity of breast cancer cell lines to clofarabine. These data when compared with the literature, suggest that resistance to clofarabine and other NAs in different cell lines is mediated by different mechanisms.

Chapter 6: The Role of ERK 1/2 in the Activity of Clofarabine in Breast Cancer Cells

6.1 Introduction

The MAPKs transduce extracellular signals with control over a variety of cellular responses that include cell growth, division, differentiation and apoptosis (Chen *et al.* 2001; Luttrell 2003; Schulte and Fredholm 2003). The ERK 1/2 pathway forms a cascade of kinases that activate each other in sequence and is amongst the most important for control of cell cycle progression, and is known to activate various proto-oncogenes (Fang and Richardson 2005). Deregulation of the ERK 1/2 pathway is associated with several human cancers including colorectal (Fang and Richardson 2005), acute leukaemia (Kim *et al.* 1999), thyroid (Shih *et al.* 2002) and breast cancer (Sivaraman *et al.* 1997; Mueller *et al.* 2000; Ma *et al.* 2001).

The importance of endogenous ERK 1/2 activity to the growth of breast cancers both *in vivo* and *in vitro* is more apparent in the more invasive and aggressive tumours, especially those deemed ER-negative. Studies have demonstrated increased ERK/MAPK activity *in vivo* in malignant breast cancers compared to normal breast tissue and benign tumours of the breast with further increases in ERK 1/2 activity in higher graded tumours (Sivaraman *et al.* 1997; Zhang *et al.* 2004). However, Milde-Langosch *et al.* (2005) found the opposite to be true *in vivo*, where high levels of phosphorylated ERK 1/2 were correlated to decreased reoccurrence and lower graded or metastatic breast cancers. *In vitro*, Chen *et al.* (2009) showed increased basal levels of phosphorylated ERK 1/2 in ER-negative tumours (MDAMB231, MDAMB436 and BT549) compared to ER-positive tumours (MCF-7, T47D and ZR-75-1). MDAMB231 cells, which exhibit high levels of constitutively activated ERK 1/2, were suggested to have increased cyclins (cyclin D1) and urokinase-type plasminogen activator expression that ultimately lead to increased proliferation and

invasiveness (Seddinghzadeh *et al.* 1999). The increased levels of ERK 1/2 signalling in ER-negative tumours are suggested to arise from overexpression of growth receptors such as EGFR that drive the proliferation of these cells (Eralp *et al.* 2008).

Increased basal ERK 1/2 signalling has been associated with increased resistance to chemotherapy (Eralp *et al.* 2008). Resistance to anti-hormones such as tamoxifen has been indicated to occur via elevated ERK 1/2 signalling (Knowlden *et al.* 2003; Cui *et al.* 2006 and Massarweh *et al.* 2008). Elevated ERK 1/2 signalling has also been associated with resistance to microtubule inhibitors (MacKeigan *et al.* 2000; McDaid and Horwitz 2001), DNA damaging agents (Hayakawa *et al.* 1999; Persons *et al.* 1999; Cui *et al.* 2000), topoisomerase inhibitors (Guise *et al.* 2001) and antimetabolites including NAs (Jarvis *et al.* 1998; Smal *et al.* 2007). The ability to block the ERK 1/2 pathway and increase sensitivity of tumours to chemotherapeutics has led to the development of new, small-molecule inhibitors of various stages in the MAPK/ERK pathway that are in clinical trials (Kohno and Pouyssegur 2006), including MEK inhibitors (AZD6624), Raf-1 inhibitors (sorafenib) and farnesyltransferase inhibitors (tipifarnib) (Grant 2008). Smal *et al.* (2007) had previously shown that pharmacological inhibition of ERK 1/2 signalling in leukaemic cells increased the efficacy of cladribine significantly probably due to the removal of the pro-survival signals by ERK 1/2 (Platanias 2003). Increased ERK 1/2 signalling is associated in a similar way with breast cancers and, in particular with ER-negative and more aggressive tumours (Sivaraman *et al.* 1997; Chen *et al.* 2009).

The anti-cancer activity of MEK inhibitors is rather limited and they have proven to be relatively unsuccessful in clinical trials in breast cancer (Mirzoeva *et al.* 2009). However, in combination with direct anti-cancer agents and other chemotherapeutic agents that are used in the treatment of cancer, their use seems to potentiate the activity of these

drugs in numerous tumour and cancer types (McDaid and Horwitz 2001; Romerio and Zella 2002; Brantley-Finely *et al.* 2003; Li *et al.* 2003b; Sun and Sinicrope 2005; Cusimano *et al.* 2007; Mirzoeva *et al.* 2009; Xu *et al.* 2009).

There is a contrast in these current data as elevated ERK 1/2 signalling is associated with resistance to chemotherapeutic agents, yet, as shown in Chapter 4, the ER-negative cells with elevated ERK 1/2 signalling are more sensitive to clofarabine and PNAs. Therefore, there is a need to understand better the role of ERK 1/2 signalling in relation to the activity of clofarabine in breast cancers.

6.2 Chapter Aims

The aim of this chapter was:

- to determine the role of ERK 1/2 in relation to the activity of clofarabine in MCF-7 and MDAMB231 breast cancer cells.

The specific objectives of this chapter were to:

- confirm basal expression of phosphorylated ERK 1/2 in MCF-7 and MDAMB231 cells.
- investigate the effects of reducing ERK 1/2 signalling in both breast cancer cell lines, and determine if this has any effect on the activity of clofarabine.
- determine if clofarabine is able to activate or reduce ERK 1/2 activity by looking for phosphorylation after treatment with clofarabine.

6.3 Methods

The detailed protocols for the Western blotting and MTS assay are described in Chapter 2.

6.3.1 Western blotting

Cell lysates were prepared from cells in logarithmic growth phase and protein quantified as described in sections 2.5.1 and 2.5.3, respectively. To determine the effects of clofarabine or the MEK inhibitor, PD 98059 (20 μM; Smal *et al.* 2007) on protein levels, cells were treated with the appropriate concentrations of the drug for 96 hours in normal growth media. Media only and vehicle controls (DMSO) were also included. Routinely, 10 μg of protein was separated using SDS-PAGE and proteins were then transferred to nitrocellulose membrane using semi-dry immunoblotting (section 2.5.5). After blocking, membranes were incubated with the primary antibody and secondary antibodies and detected using ECL. The concentrations of primary and secondary antibodies used are given below.

GAPDH	1°	1:50,000
	2°	1:50,000 anti-mouse
phospho-ERK1/2	1°	1:2000
	2°	1:10,000 anti-rabbit
total-ERK1/2	1°	1:1000
	2°	1:10,000 anti-rabbit

Densitometry was used to semi-quantitate protein levels and analysed using ImageJ software (NIH, USA). The data are expressed as the densitometry value of ERK 1 and ERK 2 combined. All data were normalised to GAPDH to control for protein loading and to ensure

consistency between the two cell lines, blots were analysed that had equal GAPDH intensity in both the MCF-7 and MDAMB231 cells.

6.3.2 MTS Assay

Cells were seeded at 5000 cells per well into 96 well plates and allowed to plate down overnight. The normal growth media was aspirated from the cells and replaced with clofarabine (0.01, 0.1 or 1 μM), PD 98059 (20 μM) or a combination of both-containing media. Relevant media only, vehicle (DMSO 0.05%) and blank controls were included on each plate. Cells were then left for 96 hours at 37°C in 5% CO_2 in a humidified atmosphere. The MTS assay was then performed as described (2.3.2).

6.3.3 Data Analysis

A two-tailed Student's *t* test was used to compare two groups. Comparisons between three or more groups were carried out using a one-way ANOVA with a Tukey-Kramer *post hoc* test if a significant difference was observed between the three or more groups. Probability values of less than 0.05 were considered statistically significant.

6.4 Results

6.4.1 Basal ERK 1/2 signalling in MCF-7 and MDAMB231 cells

Western blotting was used first to determine the basal level of ERK 1/2 signalling in MCF-7 and MDAMB231 cells. This was done by probing blots for phosphorylated ERK 1/2 (T185-T202) as this is a measure of ERK 1/2 activity, and also for GAPDH as a loading control (Figure 6.1 A). There was a significant ($p<0.01$) ~2.5-fold increase in basal levels of ERK 1/2 signalling in the MDAMB231 cells compared to the MCF-7 cells (Figure 6.1 B).

6.4.2 Effects of MEK inhibition on MCF-7 and MDAMB231 cell viability

The MTS assay was used to determine if the MEK inhibitor, PD 98059 had any significant effect on the viability of MCF-7 and MDAMB231 cells after 96 hours of treatment. Neither DMSO nor PD 98059 had any significant effect on the growth of MCF-7 cells compared to control (Figure 6.2 A). Figure 6.2 B shows that DMSO had no significant effect on MDAMB231 cell growth compared to control. At 20 μM, however, PD 98059 significantly ($p<0.001$) reduced viability to 82.4±1.0% of control.

Western blotting was then used to confirm that PD 98059 had reduced ERK 1/2 phosphorylation. Figures 6.2 C and D show that PD 98059 clearly reduced the amount of phosphorylated but not total ERK 1/2 in MCF-7 and MDAMB231 cells, respectively. GAPDH was used to confirm protein loading.

6.4.3 Effects of MEK inhibition on clofarabine activity

Next, the effects of 20 μM PD 98059 on the activity of clofarabine were determined with the MTS assay in MCF-7 and MDAMB231 cells and are shown in figures 6.3 A and B, respectively. The data in these figures are expressed as a % of their respective controls. Clofarabine alone is as a % of control (media only) treated cells.

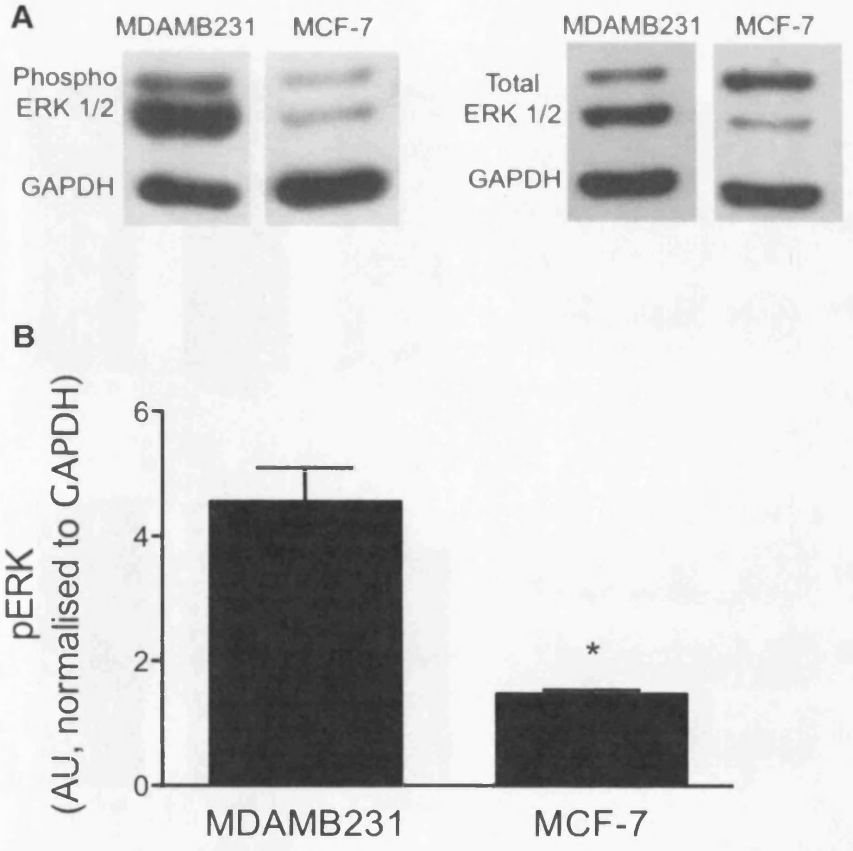


Figure 6.1 Basal Levels of Phosphorylated ERK1/2 in MCF-7 and MDAMB231 Cells

*The basal levels of phosphorylated ERK 1/2 were determined from MCF-7 and MDAMB231 cells in logarithmic growth by Western blot. Representative blots are shown (A) where 10 µg total protein was subjected to SDS-PAGE electrophoresis, blotted and probed for phosphorylated ERK 1/2, total ERK 1/2 and GAPDH. Densitometry on scanned images were used to semi-quantitate the levels of phosphorylated ERK 1/2, and data were normalised to GAPDH (B). Data are expressed as the mean of three separate experiments ± S.E.M. * significantly (p=0.005) different to MDAMB231 cells analysed with a two-tailed Student's t test.*

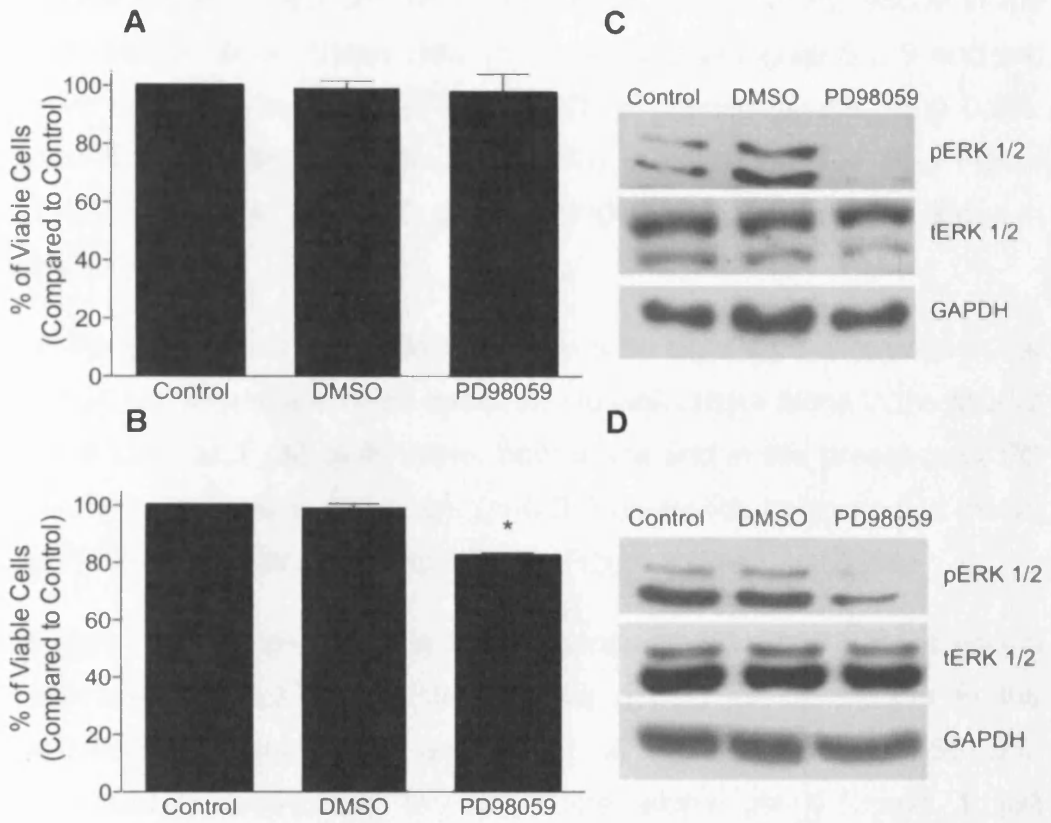


Figure 6.2 Effects of the MEK Inhibitor, PD98059 on MCF-7 and MDAMB231 Viability

*MCF-7 (A) and MDAMB231 (B) cells were incubated with media only, 0.05% DMSO or 20 μM PD 98059 for 96 hours and cell viability was assessed with the MTS assay. MCF-7 (C) and MDAMB231 (D) cells treated as for A and B were lysed and 10 μg of total protein was subjected to SDS-PAGE electrophoresis, blotted and probed for phosphorylated ERK 1/2 (pERK 1/2), total ERK 1/2 (tERK 1/2) and GAPDH. The MTS data are expressed as the mean of three separate experiments repeated in triplicate ± S.E.M. Western blot data are from a single experiment. * significantly ($p<0.001$) different to control cells analysed by one-way ANOVA with Tukey-Kramer post hoc test.*

Clofarabine in the presence of PD 98059 is expressed as a % of PD 98059 alone, to account for the significant effects of PD 98059 in the MDAMB231 cells. These data are presented in Figure 6.2 B and are from the same experiment. As DMSO, at concentrations up to 0.5%, had no significant effect on cell viability in either cell line (see Figure 4.8, Chapter 4.4), a DMSO plus PD 98059 control was not included in the assay.

In the presence of PD 98059, there was no significant difference in the activity of clofarabine when compared to clofarabine alone in the MCF-7 cells. Only at 1 μM clofarabine, both alone and in the presence of PD 98059 was there a significant ($p<0.01$) difference compared to media and PD 98059 controls, respectively (Figure 6.3 A).

Figure 6.3 B shows that in the presence of PD 98059, there was a significant ($p<0.001$) reduction in the activity of clofarabine in the MDAMB231 cells at 0.1 and 1 μM of 16.2±2.3% and 15.5±1.3%, respectively, compared to clofarabine alone. At 0.1 and 1 μM clofarabine, both alone and in the presence of PD 98059, there was a significant reduction ($p<0.001$) in viable cells compared to media and PD 98059 controls, respectively.

6.4.4 Effects of clofarabine on ERK 1/2 phosphorylation

Finally, Western blotting was used to determine if clofarabine affected the phosphorylation of ERK 1/2 in MCF-7 and MDAMB231 cells. Figures 6.4 A and B show representative Western blots of the effects of clofarabine on phosphorylated and total ERK 1/2 in the MCF-7 and MDAMB231 cells, respectively. Figures 6.4 C and D shows the results of densitometry analysis of phosphorylated ERK 1/2 levels in MCF-7 and MDAMB231 cells, respectively. Clofarabine had no significant effect on ERK 1/2 phosphorylation in either MCF-7 or MDAMB231 cells.

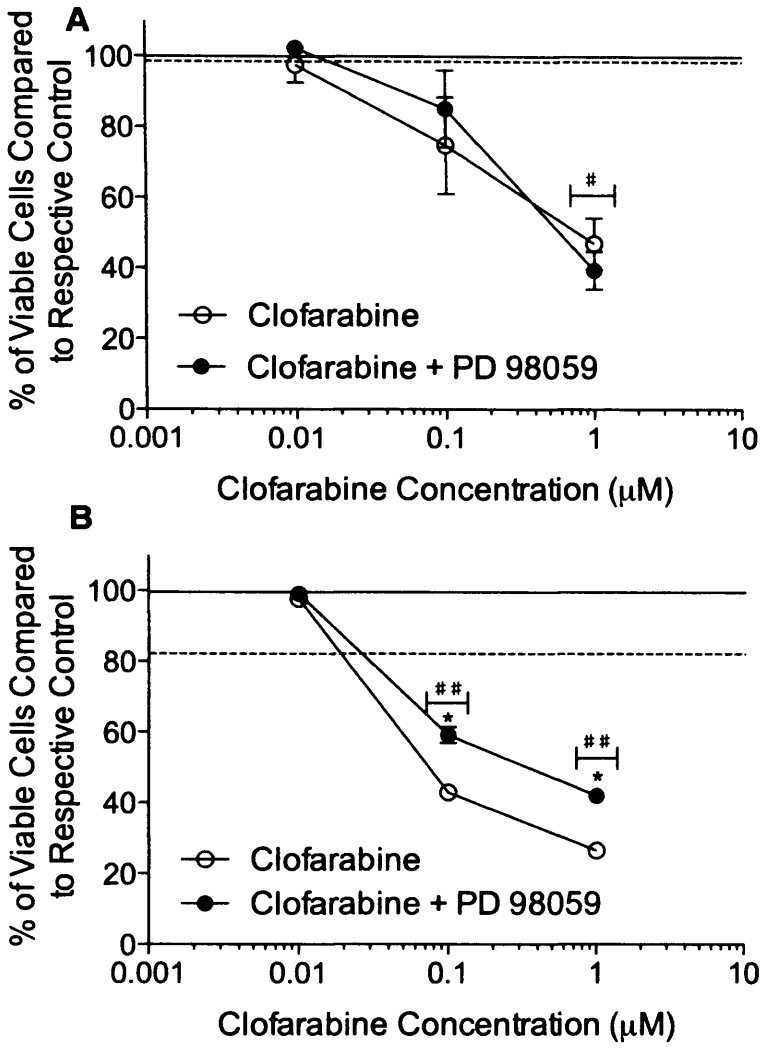


Figure 6.3 Effects of the MEK inhibitor, PD 98059 on the Activity of Clofarabine on Cell Viability of MCF-7 and MDAMB231 Cells

The effect of the PD 98059 (20 μM) on the activity of clofarabine on cell viability after 96 hours was determined by the MTS assay in MCF-7 (A) and MDAMB231 (B) cells. Solid line represents DMSO (0.005%), dashed line represents 20 μM PD 98059 alone. Data are expressed as the mean of three separate experiments repeated in triplicate ± S.E.M. $^{\#}(p<0.01)$, $^{\#\#}(p<0.001)$ significantly different compared to control. $^{*}(p<0.001)$ significantly different compared to clofarabine alone. Data analysed with two-way ANOVA followed by Bonferroni post hoc test. Clofarabine alone data are expressed as % of media (100%) control. Clofarabine + PD 98059 data are expressed as a % of PD 98059 alone.

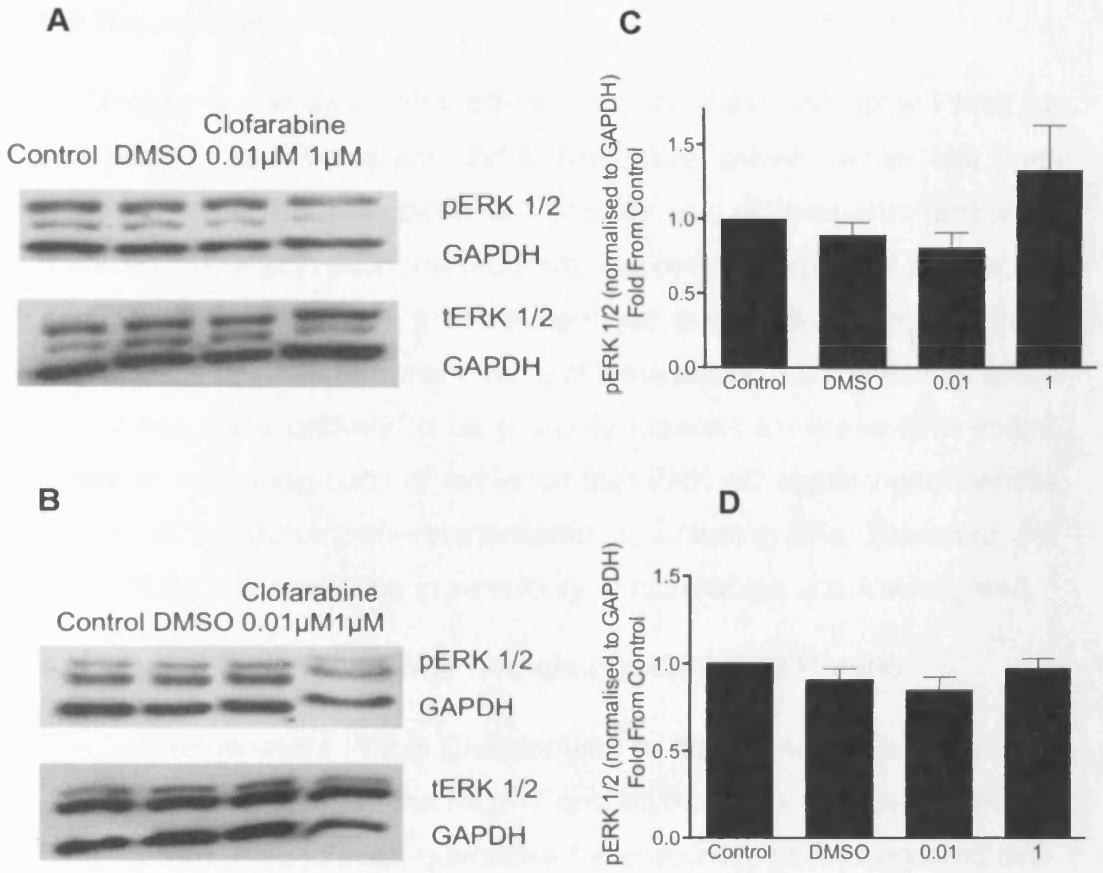


Figure 6.4 Effects of Clofarabine on the Phosphorylation of ERK1/2 in MCF-7 and MDAMB231 Cells

Levels of phosphorylated ERK 1/2 were determined by Western blot in MCF-7 (A and C) and MDAMB231 (B and D) cells after treatment with media alone (control), 0.05% DMSO, 0.01 μM or 1 μM clofarabine for 96 hours. Representative blots are shown (A and B, MCF-7 and MDAMB231, respectively) where 10 μg total protein was subjected to SDS-PAGE electrophoresis, blotted and probed for phosphorylated ERK 1/2 (pERK 1/2), total ERK 1/2 (tERK 1/2) and GAPDH. Densitometry on scanned images were used to semi-quantitate the levels of pERK 1/2, and data were normalised to GAPDH (C and D, MCF-7 and MDAMB231, respectively). Data are expressed as the mean of three separate experiments ± S.E.M. No significant differences were detected with one-way ANOVA.

6.5 Discussion

In Chapter 4, the differential effects of clofarabine and other PNAs on cell viability in ER-positive and ER-negative breast cancer cell lines were identified. In the subsequent Chapter, the expression of enzymes involved in the activation, metabolism and cellular targets of clofarabine was investigated. These studies identified a conceivable role of cN-II and p53R2 in the differential effects of clofarabine, but it was suggested that these were unlikely to be the only reasons for these differences. There is a growing body of evidence that ERK 1/2 signalling influences sensitivity to cytotoxic chemotherapeutics, including NAs. Therefore, the role of ERK 1/2 signalling in sensitivity to clofarabine was investigated.

6.5.1 Basal ERK 1/2 activity is higher in MDAMB231 cells

The first experiment in this Chapter was to determine the basal activity of ERK 1/2 signalling in the MCF-7 and MDAMB231 cell lines. Western blotting was used to semi-quantitate the amount of phosphorylated ERK 1/2 in both these cell lines, lysed during normal logarithmic growth in culture. These experiments showed clearly that the MDAMB231 cells have significantly more phosphorylated ERK 1/2 compared to the MCF-7 cells by ~2.5-fold. The amount of GAPDH in each cell line was fairly consistent which indicates that there was an equal amount of protein loaded and thus a fair comparison can be made.

Several other studies have identified an increase in basal ERK 1/2 activity in MDAMB231 and ER-negative cell lines compared to MCF-7 and other ER-positive cell lines in culture (Krueger *et al.* 2001; Ogata *et al.* 2001; Chen *et al.* 2009). Creighton *et al.* (2006) suggested that increased expression of ERK 1/2 causes loss of ERα, and the generation of an ER-negative phenotype. This elevated ERK 1/2 activity is often due to increased receptor tyrosine kinase expression including epidermal growth factor receptor (EGFR) and Her2 (Eralp *et al.* 2008; Toyama *et al.* 2008). The data herein are in agreement with the

literature and therefore help validate the subsequent experiments described in this Chapter.

An interesting study by Piao and colleagues found that p53R2 interacted directly with MEK2, an upstream activator of ERK 1/2. Their study identified the role of p53R2 in suppressing MEK2 and ERK 1/2 activity in lung cancer cell lines (Piao *et al.* 2009) and hypothesised that p53R2 expression might suppress the mitogenic potential of tumours. In the previous chapter, p53R2 expression was found to be higher in ER-positive tumours, and this is correlated with lower basal ERK 1/2 signalling.

These data also suggest that increased basal ERK 1/2 signalling does not correlate with increased resistance to PNA in these breast cancer cell lines. ERK 1/2 signalling is associated with an increase in pro-survival and transcription factor activation (Chen *et al.* 2001), and this increase has been shown to 'protect' cells against a variety of clinically used anti-cancer drugs including cisplatin, vinblastine, vincristine, paclitaxel, cladribine, doxorubicin and cytarabine (Jarvis *et al.* 1998; Hayakawa *et al.* 1999; Persons *et al.* 1999; Cui *et al.* 2000; MacKeigan *et al.* 2000; Guise *et al.* 2001; McDaid and Horwitz 2001; Stadheim *et al.* 2001; Brantley-Finley *et al.* 2003; Smal *et al.* 2007). These studies suggested that inhibition of the pro-survival ERK 1/2 signalling pathway 'primed' cells for apoptosis, enhancing the effects of these commonly used cytotoxic agents that included DNA-damaging agents, anti-metabolites, topisomerase inhibitors and microtubule inhibitors. Similarly, Normanno *et al.* (2006) found that resistance to the tyrosine kinase inhibitor gefitinib, was directly related to an increase in MEK/ERK 1/2 activity in MDAMB468 cells in particular. They found that inhibition of ERK 1/2 by PD 98059 synergistically increased the action of gefitinib. This was also seen in SKBR-3 and MDAMB361 cell lines, although only with an additive effect.

However, the increase in basal ERK 1/2 signalling herein is associated with increased sensitivity to clofarabine and other PNA and is discussed in the following sections.

6.5.2 Pharmacological inhibition of ERK 1/2 decreases the activity of clofarabine in MDAMB231 cells

The previous experiment demonstrated that increased basal ERK 1/2 signalling is not associated with increased resistance to PNA in MCF-7 or MDAMB231 cells. However, this does not rule out the involvement of ERK 1/2 signalling in the activity of clofarabine or other PNA. The following experiments were designed to try and answer the question as to how or if ERK 1/2 signalling was involved in the action of clofarabine in the MCF-7 and MDAMB231 cell lines.

Initial studies characterised the response of the MEK inhibitor, PD 98059, on the viability of MCF-7 and MDAMB231 cells in culture. MEK is an upstream kinase to ERK 1/2 that is directly involved in the phosphorylation of ERK 1/2. Therefore, through its inhibition, ERK 1/2 phosphorylation is proportionally reduced. PD 98059 is widely used for this purpose as it does not inhibit other protein kinases at the concentration used here (Davies *et al.* 2000).

20 μM PD 98059 had no effect on the viability of MCF-7 cells. This suggests that ERK 1/2 signalling is not a key pathway for normal cell functioning in these cells. However, PD 98059 significantly decreased the amount of viable MDAMB231 cells by 20% compared to control suggesting a role for ERK 1/2 signalling in these cells which has been found previously (Seddighzadeh *et al.* 1999). This notion is supported by the elevated basal ERK 1/2 signalling observed in the MDAMB231 cells, compared to the MCF-7 cells. In order to confirm that this effect on cell viability was due to decreased ERK 1/2 signalling, Western blotting was used to confirm ERK 1/2 knockdown following treatment with PD 98059. A substantial, albeit not maximal, decrease in

phosphorylated ERK 1/2 in both MCF-7 and MDAMB231 cells was seen.

To determine the role that ERK 1/2 was playing in relation to clofarabine activity, MCF-7 and MDAMB231 cells were incubated with several concentrations of clofarabine alone, or clofarabine in combination with PD 98059. In the MCF-7 cells, no difference was observed between these two treatment schedules at any concentration strongly indicating that reduction of ERK 1/2 signalling has no bearing on the potency of clofarabine. As ERK 1/2 basal activity is minimal in MCF-7 cells, this strongly suggests that it is a relatively under-utilised pathway in normal cell functions, at least *in vitro*. ERK 1/2 signalling in ER-positive cells is not to be overlooked, however, as it has previously been demonstrated in ER-positive tumours that binding of estrodiol to the ERα receptor at the cell membrane can directly induce MAPK activation (Santen *et al.* 2002) and data have shown that this increase in phosphorylated ERK 1/2 can result in increased cellular proliferation (Peralta *et al.* 2006).

It is interesting to look at what effects increased ERK 1/2 signalling had on the MCF-7 cells. Although ERK 1/2 may not be involved in pro-survival 'per se' here, an increase in activity may affect clofarabine potency. Studies have identified an increase in ERK 1/2 signalling in breast cancer cells resistant to anti-hormones. Knowlden *et al.* (2003), Cui *et al.* (2006) and Massarweh *et al.* (2008) showed that MCF-7 cells made resistant to tamoxifen (TamR) had an increase in ERK 1/2 signalling compared to their wild-type parental cell lines and that this signalling was important in cell growth. This feature has also been demonstrated with fulvestrant-resistant cells (Frogne *et al.* 2009). Testing clofarabine against these cell lines may give an insight into this possibility.

In the MDAMB231 cells in the presence of PD 98059, clofarabine activity was decreased compared to clofarabine alone at concentrations of 0.1 and 1 μM by ~15%. This suggests that ERK 1/2 signalling

accounts for part of the mechanism behind the anti-cancer activity of clofarabine in these cells and that ERK 1/2 sensitizes cells to the action of clofarabine for the cytotoxic action. Similar findings have identified the role of ERK 1/2 in sensitizing cells to other cytotoxic agents. Cisplatin-induced apoptosis was decreased following treatment of HeLa ovarian carcinoma cells with PD 98059 leading to reduced ERK 1/2 signalling (Wang *et al.* 2000). They found that ERK 1/2 was initiating the apoptotic signal upstream of cytochrome C release and caspase activation. Also, Bacus *et al.* (2001) showed that paclitaxel-induced apoptosis was significantly reduced following ERK 1/2 phosphorylation inhibition by PD 98059 in MCF-7 cells. They found that paclitaxel induced G2/M cell cycle arrest and apoptosis, but in PD 98059 pre-treated cells, paclitaxel only induced cell cycle arrest in the G2/M phase. This suggests that ERK 1/2 is involved in initiating the apoptotic cascade, with similar results suggested by Guise *et al.* (2001) in neuroblastoma cells treated with paclitaxel and doxorubicin. Similar results have also been shown with cisplatin and doxorubicin using renal epithelial cells, also identifying ERK 1/2 signalling upstream of Bax activation, cytochrome C release and caspase activation (Alexia *et al.* 2004; Arany *et al.* 2004; Kim *et al.* 2005).

The reasons for this paradoxical ERK 1/2 signalling as an apoptosis inducer are unknown here. Although ERK 1/2 signalling is important for the growth of MDAMB231 cells, it may have dual functions. Most literature suggests ERK 1/2 as a pro-survival kinase (Chen *et al.* 2009), but studies have also suggested its role as an apoptosis inducer (Mohr *et al.* 1998; Blagosklonny 1998). Indeed Ishikawa and Kitamura (1999) suggested a dual-functioning role for ERK 1/2 as both necessary at basal constitutional levels in normal cell functioning, but temporary increases in ERK 1/2 signalling were involved in induction of apoptosis.

Clofarabine did not affect ERK 1/2 signalling directly, as active concentrations of clofarabine (1 μM) failed to increase or decrease

levels of phosphorylated ERK 1/2 compared to controls in either MCF-7 or MDAMB231 cells. One of the reasons for this could be due to the methods used. Western blotting using antibodies directed against a phosphorylation site is a widely used method for looking at the activation of signalling molecules. However, due to the high level of basal ERK 1/2 signalling in the MDAMB231 cells, a further increase in phosphorylation of ERK 1/2 may be missed by saturation of the assay. One way to overcome this would be to reduce basal activity of ERK 1/2 by removal of exogenous growth factors and quiescing the cells. Although this method can be used to look at ERK 1/2 signalling over short time periods (see Chapter 3), it cannot be used for long periods here (over 96 hours) as the cells cannot cope with this deprivation of growth factors and die. However, clofarabine did not induce ERK 1/2 phosphorylation in the MCF-7 cells, where basal levels are low. This suggests that clofarabine does not activate ERK 1/2 signalling directly, and that saturation of the assay was not masking the effects in the MDAMB231 cells. Methods such as ELISA are more sensitive and may be able to pick up the changes in ERK 1/2 phosphorylation more accurately and therefore could be used to confirm the data here.

Studies by Smal *et al.* (2007) and Conrad *et al.* (2009) both found that cladribine was able to induce ERK 1/2 phosphorylation in leukaemia cell lines. Fernández-Calotti *et al.* (2006) was also able to demonstrate that fludarabine could activate ERK 2 in leukaemic cells. Pyrimidine NAs have also been demonstrated to increase ERK 1/2 phosphorylation including gemcitabine (Matsumoto *et al.* 2008), ganciclovir (Whartenby *et al.* 2002) and cytarabine (Jarvis *et al.* 1998; Stadheim *et al.* 2001).

Similarly, numerous other studies have found that other cytotoxics can induce phosphorylation of ERK 1/2 in cell lines that include MCF-7 and MDAMB231 cells, including doxorubicin, epirubicin cisplatin, ceramide, etoposide and paclitaxel (Hayakawa *et al.* 1999; Persons *et al.* 1999; Cui *et al.* 2000; MacKeigan *et al.* 2000; Bacus *et al.* 2001; Guise *et al.*

2001; McDaid and Horwitz 2001; Boldt *et al.* 2002; Small *et al.* 2003). However, other studies have found that the same cytotoxics can decrease ERK 1/2 signalling in other cancer cell types (Amato *et al.* 1998; Stone and Chambers 2000; Stadheim *et al.* 2001).

These data suggest that, as inhibition of ERK 1/2 signalling reduces clofarabine efficacy in MDAMB231 cells, and clofarabine does not affect ERK 1/2 signalling directly, one of the mechanisms behind clofarabine activity lies at a post-ERK 1/2 phosphorylation site. As suggested, ERK 1/2 may be involved in the upstream activation of apoptosis (Ishikawa and Kitamura 1998; Wang *et al.* 2000; Zhuang and Schnellmann 2006) as a consequence of the cytotoxic mechanism of clofarabine. However, as clofarabine did not increase ERK 1/2 phosphorylation, this may suggest that the DNA damage caused by clofarabine may be affecting an intracellular target that relies on, and is downstream of ERK 1/2. Therefore, through ERK 1/2 inhibition, this target is no longer activated and less cell death is observed. Arany *et al.* (2004) showed that ERK 1/2 inhibition inhibited caspase 3 activation, an effector caspase in the intrinsic apoptosis pathway. MCF-7 cells are known to be caspase 3-null, whereas MDAMB231 cells are not (Yang *et al.* 2007), which may explain the differences seen with PD 98059 and clofarabine treatment. This may then also explain why an increase in ERK 1/2 phosphorylation was not observed. Similarly, Bax, a member of the Bcl-2 proteins is a pro-apoptotic protein. It is translocated to the mitochondria upon apoptotic stimuli where it causes release of pro-apoptotic proteins such as cytochrome C and the activation of caspases (Chiu *et al.* 2003). Exposure to cisplatin or reactive oxygen species has been shown to increase Bax expression, consistent with apoptosis, but in the presence of ERK 1/2 signalling, a decrease in Bax is observed (Kim *et al.* 2005; Park *et al.* 2005).

A potential mechanism of ERK 1/2 signalling and its role in clofarabine-induced cell death is shown below (figure 6.5.1).

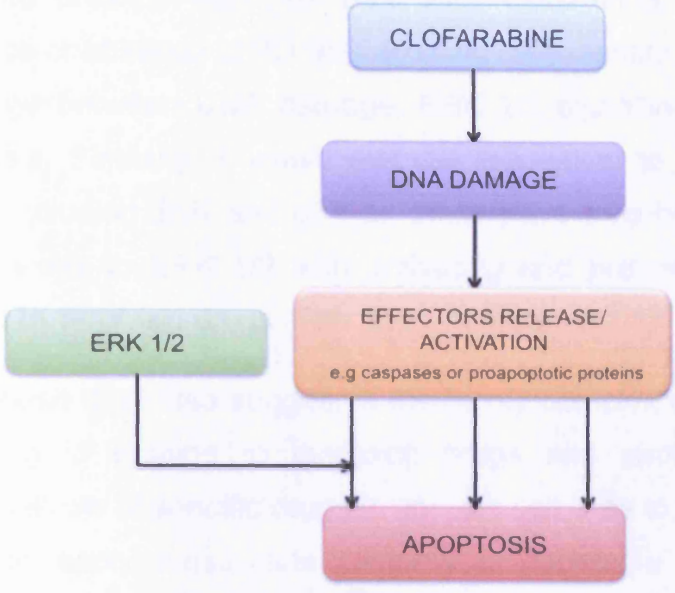


Figure 6.5 Representation of the Role of ERK 1/2 Signalling in Clofarabine-Induced Apoptosis in MDAMB231 Breast Cancer Cells

Clofarabine causes DNA damage, which leads to the initiation of apoptosis by the release or activation of apoptosis-inducing proteins. Some of these require either activation by ERK 1/2, or interaction with an ERK 1/2-dependent molecule. This then leads to apoptosis. Reduction in ERK 1/2 signalling removes this process therefore reducing apoptosis, although other processes that are ERK 1/2-independent can still cause significant cell death.

These data suggest that breast cancer cell lines, at least *in vitro*, with higher basal ERK 1/2 activity are more susceptible to the actions of clofarabine. ERK 1/2 signalling, at least in part, appears to sensitize the cells to the cytotoxic action of clofarabine, and removal of the ERK 1/2 pathway reduces the efficacy of clofarabine in MDAMB231 cells. It would be interesting to look at ERK 1/2 signalling in other breast cancer cell lines in relation to clofarabine efficacy and see if correlations appear with basal activity or inhibition of ERK 1/2 signalling. It would also be interesting to look at the precise mechanism by which ERK 1/2 signalling is partly mediating clofarabine-induced apoptosis in MDAMB231 cells. Looking at the expression of caspases and pro-

apoptotic proteins such as Bax after clofarabine treatment in the presence or absence of PD 98059 would help identify the key players at the stage between DNA damage, ERK 1/2 signalling and initiation of apoptosis. Similarly, it would also be interesting to look at the other MAPK including JNK and p38 as these have also been associated in similar ways to ERK 1/2 with activating and preventing apoptosis in relation to cytotoxic drugs (Fan and Chambers 2001).

What these data also suggest is the highly complex nature of ERK 1/2 signalling in relation to cytotoxic drugs and strongly indicate the individual role of specific drugs in specific cell lines to this pathway. The ability to apply these data clinically is debatable due to the wide differences in ERK 1/2 signalling in normal tissues, and in different cancers. Gee *et al.* (2001) demonstrated a decrease in disease-free survival in breast tumours deemed positive for phosphorylated ERK 1/2 compared to those deemed ERK 1/2-negative after the initiation of anti-hormone therapy. Derin *et al.* (2008) also found that low levels of ERK 1/2 signalling in ER-negative breast cancer were associated with anthracycline resistance and shorter survival rates. Interestingly, they found that those people with higher ERK 1/2 signalling also had shorter survival rates compared to reduced ERK 1/2 signalling after disease reoccurrence. These data may suggest a differential role of ERK 1/2 at different stages of tumour progression clinically. Interestingly, Boldt *et al.* (2002) identified no particular correlation with drug-induced MAPK activity and efficacies of cytotoxic drugs alone, or in combination with MAPK inhibitors. Their data suggest that MAPK inhibition can either increase or decrease the efficacy of cytotoxic drugs, and that the potential for using MAPK inhibitors would have to tailored to individual tumours, a view supported here.

6.6 Conclusion

The data herein suggest there is no direct relationship between ERK 1/2 signalling and clofarabine in the MCF-7 cells. However, these data do suggest that in MDAMB231 cells increased basal ERK 1/2 signalling is a feature that promotes the anti-cancer activity of clofarabine, and reducing ERK 1/2 signalling reduces the efficacy of clofarabine against these cells. The mechanism by which this is occurring was not elucidated in this study due to time constraints. As clofarabine did not alter ERK 1/2 signalling directly it is possible that clofarabine activity is partially dependent upon ERK 1/2 activity on another cellular process. Further work is therefore needed to help characterise the mechanism by which ERK 1/2 is related to the cytotoxic activity of clofarabine.

Clinically, these data suggest that inhibition of ERK 1/2 signalling may not be of clinical benefit when combined with cytotoxic chemotherapy, specifically with clofarabine. Although alone, ERK 1/2 inhibition did produce a small effect against MDAMB231 cells, it is unlikely that this has clinical potential as it was such a small effect. Looking at the relationship between ERK 1/2 signalling, and the efficacy of clofarabine could be used in the future to predict how likely a patient is to respond to clofarabine treatment. Further understanding of the molecular interplay between ERK 1/2 signalling and clofarabine efficacy could lead to development of novel therapies or treatment strategies to exploit these pathways.

Chapter 7: General Discussion

7.1 Discussion

Breast cancer is a group of heterogeneous neoplasms that arise from the breast and is now the most common form of cancer in the UK, with an annual incidence of 45,000 new cases. Worldwide, there are over one million new cases of breast cancer diagnosed annually, with the highest incidences occurring in North America (Cancer Research UK, 2010). Despite falling death rates over the last 10 years, 12,000 people still die annually from breast cancer in the UK and it is now the second most common form of death from cancer after lung cancer in women (Cancer Research UK, 2010).

Current treatments for breast cancer depend upon the molecular status of the cancer, specifically whether they express ER and/or PR, and also the expression level of the Her2 receptor. Other factors are also taken into account such as age, general health and menopausal status. For those women with cancers that are ER-positive and/or Her2 overexpressed, effective targeted therapies are available i.e. aromatase inhibitors and trastuzumab, respectively. TNBC, however, accounts for 20% of all newly diagnosed cases of breast cancer and there are currently no targeted chemotherapy options (Skliris *et al.* 2008).

Besides surgery and radiation, cytotoxic chemotherapy forms the basis of treatment for women with TNBC. Despite good initial response, reoccurrence is high and prognosis for these women is extremely poor (Carey *et al.* 2007). There is no standardised treatment regime but generally combination therapy is used including two or three agents from the taxanes (paclitaxel), anthracyclines (doxorubicin), alkalying agents (cyclophosphamide) and antimetabolites (5-fluorouracil and methotrexate) (Anders and Carey 2008; Gluz *et al.* 2009). The major limitations to these treatments are the development of significant toxicities and side effects including nausea, fatigue, mucosal toxicity,

hair loss and neurotoxicity that severely affect quality of life. Drugs such as taxanes and anthracyclines can also cause life-threatening side effects such as heart failure and the development of leukaemia (Shapiro and Recht 2001; Nowak *et al.* 2004). As such, there is a very obvious need for the development of new treatments for TNBC that are effective and also well tolerated by patients.

The adenosine system and adenosine analogues represent attractive targets for the development of new and novel therapies. Numerous studies have identified that adenosine receptor activation inhibits the growth of various tumours, including breast (Colquhoun and Newsholme 1997; Yao *et al.* 1997; Fishman *et al.* 2000; Ohana *et al.* 2001; Merighi *et al.* 2003; Panjehpour and Karami-Tehrani 2004 and 2007; Lee *et al.* 2005; Sai *et al.* 2006; Bar-Yehuda *et al.* 2008; Kim *et al.* 2008). The adenosine receptors, particularly the A_1 and A_3 receptors have also been shown to be upregulated in cancerous tissues over normal tissues, and this has been specifically demonstrated in breast cancers (Khoo *et al.* 1996; Madi *et al.* 2004; Gessi *et al.* 2007; Ahmad *et al.* 2009). There is therefore the potential that exploitation of these receptors could lead to novel treatments.

Adenosine analogues are widely used in the treatment of haematological malignancies. They are very effective, sometimes producing curative outcomes and are also well tolerated by patients without many of the side effects commonly associated with standard cytotoxic chemotherapy (Guchelaar *et al.* 1994; Selby *et al.* 1998; Seshadri *et al.* 2000; Rummel *et al.* 2002; Cooper *et al.* 2004; Jeha *et al.* 2004). There have only been limited studies in solid tumours but their potential is evident (Takahashi *et al.* 1999; Waud *et al.* 2000; Hashemi *et al.* 2003, 2004; Wang and Albertioni 2010).

The aim of this thesis was to explore the potential of adenosine and adenosine analogues to reduce the viability of breast cancer cell lines *in vitro*. The studies were mainly carried out on the two widely used MCF-

7 (ER-positive) and MDAMB231 (ER-negative, TNBC) cell lines. Some studies were also extended using four other breast cancer cell lines to attempt to reflect the different phenotypes that exist in breast cancer.

Initial results demonstrated the expression of all four adenosine receptors in both the MCF-7 and MDAMB231 cell lines. However, antagonism of these receptors had no effect upon cell viability in either cell line suggesting endogenous activation of these receptors does not promote cell survival or proliferation. Furthermore, the non-selective adenosine receptor agonist, NECA, only reduced cell viability at concentrations far exceeding the *Ki* for adenosine receptors (between 14 and 5000-fold; Klotz *et al.* 2000) and only in the MCF-7 cells. Further studies then identified that adenosine at a high concentration (100 μM) was able to reduce cell viability in the MCF-7 cells by a mechanism partly involving adenosine receptor activation, which also caused a transient increase in ERK 1/2 phosphorylation. However, there was no effect upon adenosine receptors in the MDAMB231 cells.

These results indicate that in the MDAMB231 cells, adenosine receptors are not involved in cell viability, nor can their activation induce apoptosis or cytostasis. In the MCF-7 cells, activation of the adenosine receptors can reduce cell viability significantly suggesting therapeutic potential. The high concentrations of NECA and adenosine needed to reduce viability, however, suggest that they would be of little clinical benefit due to the wide distribution and functions of adenosine receptors physiologically (Klinger *et al.* 2002; Borowiec *et al.* 2006). There are selective adenosine receptor agonists available that are more potent than NECA which may offer more feasible clinical options (Jacobson and Gao 2006). Indeed, the selective A_3 receptor agonists CF101 (*Ki* = 1.1 nM) and CF102 are in phase I and II clinical trials for the treatment of colorectal cancer and hepatocellular carcinoma, alone and in combination with other cytotoxic drugs and are showing promising results with very limited side effects (Stemmer *et al.* 2004; Stemmer

2009). Due to the increased expression of the A_3 receptor on breast cancer cells over normal breast tissue (Madi *et al.* 2004), this may be a feasible target in breast cancer also. However, further work is needed to elucidate which receptor NECA and adenosine were activating.

Elimination of endogenous adenosine and 2'-deoxyadenosine with ADA caused a significant decrease in cell viability in both cell lines that was probably due to depletion of important precursor molecules such as ATP or cAMP. Localised elimination of adenosine and 2'-deoxyadenosine from the tumour microenvironment could be a useful therapy for solid tumours. Although it has been shown that gene therapy can restore ADA activity in ADA-deficient mice (Mortellaro *et al.* 2006), it is unknown whether overexpression could be induced in humans, and specifically in tumour cells.

Inhibition of adenosine metabolism by the clinically used agent, deoxycoformycin did not influence cell viability in either cell line. Although deoxycoformycin is effective in the treatment of haematological malignancies (Dillman 2004), it does not appear to be as effective in the treatment of solid tumours, but this may be a consequence of the *in vitro* methods used. Ogawa *et al.* (2000) demonstrated that, whilst deoxycoformycin was very effective in treating hairy cell leukaemia in a patient, *in vitro* deoxycoformycin had no effect upon isolated leukaemia cells. When 10 μM 2'-deoxyadenosine was introduced, however, this combination of drugs had a significant effect upon reducing cell viability suggesting a source of 2'-deoxyadenosine other than the tumour cells is needed. This theory was tested here by the addition of adenosine and 2'-deoxyadenosine in the presence of deoxycoformycin which produced significant reductions in cell viability in both cell lines. The concentrations of adenosine and 2'-deoxyadenosine in plasma after treatment with deoxycoformycin are in the order of 13 and 27 μM, respectively (Venner *et al.* 1981). In solid tumours, adenosine levels peak at 13 μM in the presence of deoxycoformycin

and adenosine kinase inhibitors (Blay *et al.* 1997). As concentrations greater than these are needed here to influence cell viability significantly in the MCF-7 and MDAMB231 cells, deoxycoformycin is unlikely to be useful against breast cancer tumours clinically.

The mechanisms of action of adenosine and 2'-deoxyadenosine were investigated next, as these compounds could offer new pathways to exploit as treatments for breast cancer. In the MCF-7 cells adenosine was working by activation of adenosine receptors and also by intracellular phosphorylation by adenosine kinase. In the MDAMB231 cells, however, the mechanism of action of adenosine was by neither of these mechanisms. Both adenosine and 2'-deoxyadenosine in the MDAMB231 cells were able to reduce ERK 1/2 phosphorylation in an adenosine receptor-independent manner, which may account for some of the effects on cell viability. However, in Chapter 6, it was demonstrated that the MEK inhibitor PD 98059 had a lesser effect on cell viability than adenosine or 2'-deoxyadenosine did here. This suggests that alternative mechanisms are also induced.

In both the MCF-7 and MDAMB231 cells, 2'-deoxyadenosine reduced cell viability by a mechanism requiring the action of adenosine kinase and this has been described previously in breast, colon and gastric cancer cell lines (Camici *et al.* 1995; Giannecchini *et al.* 2003; Hashemi *et al.* 2005). The phosphorylation of 2'-deoxyadenosine to the mono- and ultimately the triphosphate is most likely mediating these effects. Indeed, dATP has been shown to cause dNTP imbalance by inhibition of ribonucleotide reductase causing base pair mismatching in DNA and ultimately leading to apoptosis (Tanaka *et al.* 1994; Wakade *et al.* 1995; Fidias *et al.* 1996; Barry and Lind 2000; Hashemi *et al.* 2005).

The intracellular action of 2'-deoxyadenosine (with deoxycoformycin), however, did suggest that stable analogues of this compound already in the clinic could be cytotoxic to these breast cancer cell lines and so their activity was investigated next.

The clinically used PNA clofarabine and cladribine had nM potency against a range of breast cancer cell lines. Fludarabine was less active with IC_{50} values in the μM range in MCF-7 and MDAMB231 cells. Clofarabine was able to induce apoptosis in the MCF-7 and MDAMB231 cell lines at a clinically effective dose (1 μM) confirming its cytotoxic effects. All three drugs require intracellular phosphorylation by dCK, consistent with the literature (Parker *et al.* 1991; Lotfi *et al.* 1999; Månsson *et al.* 1999; Galmarini *et al.* 2001). Clofarabine and cladribine were shown to work primarily through ribonucleotide reductase inhibition whereas fludarabine was less dependent upon inhibition of this enzyme. These results are consistent with the differential potencies of these drugs for inhibiting this enzyme (Parker *et al.* 1991) and could explain the differential effects of these drugs on cell viability. Phosphorylation by dCK and transport across the membrane were identified as not being rate-limiting steps in the action of clofarabine and cladribine using phosphoramidate analogues.

Clofarabine is the most promising of these agents based upon its potency here and also preferential pharmacodynamics including increased oral bioavailability, increased resistance to degradation/metabolism, increased cellular retention and its inhibitory action on both DNA polymerase and ribonucleotide reductase (Tseng *et al.* 1982; Parker *et al.* 1991; Zhenchuk *et al.* 2009). Clofarabine was therefore the focus of subsequent studies.

Interestingly, these results demonstrated a differential effect of the PNA between ER-positive and ER-negative breast cancers, with the drugs being significantly more potent in the latter. Most interestingly, however, was that clofarabine was equally potent in the ER-negative cells compared to the HL60 leukaemia cell line in this experimental protocol. As clofarabine is used successfully against leukaemia (Robak and Wierzbbowska 2009), this strongly demonstrates its potential for its clinical application in this difficult to treat phenotype of breast cancer.

As clofarabine is effective even as a low dose regime against acute leukaemia (Bonate *et al.* 2006; Choi and Foss 2006), the excellent potency of clofarabine against the ER-negative cells suggests that low doses may be sufficient to reduce tumour burden whilst further reducing potential side-effects. The excellent potency also highlights its potential use in combinational therapy.

Whilst the ER-positive cells were less sensitive to the effects of clofarabine, it may still be useful as an adjuvant therapy for this type of breast cancer as potency was in the 300 nM range. Also, the efficacy of clofarabine at 1 μM in the T47D (ER-positive, Her2 normal expressed, p53-mutant) cells was equal to that of the leukaemia cells, suggesting that this phenotype may gain benefit from clofarabine treatment. This may be useful in cases of metastatic or hormone-resistant breast cancer, which almost always occurs upon disease reoccurrence after treatment of patients with advanced disease with anti-hormones (Goss and Strasser 2002).

Investigating the mechanisms behind the differential effect of clofarabine in ER-negative and ER-positive breast cancer cells revealed that these differences were not artefacts of cellular proliferation rates.

RT-PCR and Western blotting studies focused on investigating the expression of the activating and metabolising enzymes dCK and cN-II, respectively, and ribonucleotide reductase as these enzymes are frequently implicated in the development of resistance (Carson *et al.* 1991; Kawaski *et al.* 1993; Bai *et al.* 1998; Schirmer *et al.* 1998; Dumontet *et al.* 1999; Lotfi *et al.* 1999 and 2001; Månsson *et al.* 1999 and 2003; Gourdeau *et al.* 2001; Davidson *et al.* 2004; Jordheim *et al.* 2005; Yamamoto *et al.* 2007; Ferrandina *et al.* 2010).

Results indicated that increased basal levels of the metabolising enzyme cN-II were correlated with a decrease in the potency of clofarabine. The expression of dCK, however, was not correlated with

clofarabine potency. This is most likely due to increased metabolism of clofarabine triphosphate to the inactive forms. This may have clinical implications and be able to serve as a prognostic indicator as to which patients are most likely to respond to clofarabine treatment as suggested previously (Galmarini *et al.* 2001; Galmarini *et al.* 2003b). Similarly, cN-II may serve as a therapeutic target for pharmacological inhibition to potentiate the action of PNAs.

There was also a correlation between increased expression of p53R2 and decreased potency of clofarabine at the RNA level, and this was also confirmed at the protein level in several cell lines. Interestingly, after exposure to clofarabine, p53R2 expression was increased in the p53-wild type MCF-7 cells, but not in the p53-mutant T47D or MDAMB231 cell lines. These data strongly indicate that both basal and drug-induced expression of p53R2 can influence clofarabine potency in this panel of breast cancer cell lines. Although the mechanism behind these effects was not investigated here, it is probable that clofarabine-mediated DNA damage can induce p53R2 expression, which is able to produce dNTPs to repair the damage and out-compete clofarabine for incorporation into DNA (Tanaka et al. 2000; Barber *et al.* 2005; Tsai *et al.* 2006; Link *et al.* 2008).

Interestingly, 80% of TNBC are associated with mutated or null p53 function (Sørlie *et al.* 2001). Whilst p53 mutant tumours have generally been regarded as more resistant to cytotoxic chemotherapy, the activity of clofarabine may favour p53 mutants due to its role in inducing p53R2 in p53 wild-type cells.

Previous studies had shown that increased basal ERK activity was associated with resistance to various cytotoxic agents, including NAs (Jarvis *et al.* 1998; Hayakawa *et al.* 1999; Persons *et al.* 1999; Cui *et al.* 2000; MacKeigan *et al.* 2000; McDaid *et al.* 2001; von Giese *et al.* 2001 Smal *et al.* 2007; Eralp *et al.* 2008). This was interesting as ER-

negative cells are associated with an increase in basal ERK 1/2 activity and clofarabine was more potent in these cells.

The results here show that ERK 1/2 inhibition reduces, although not completely, the activity of clofarabine in the MDAMB231 cells but clofarabine does not influence ERK 1/2 phosphorylation directly. The exact mechanism by how this occurs was not investigated further, it is possible that ERK 1/2 is involved upstream of activating process needed for apoptosis which has been shown previously (Blagosklonny et al. 1998; Ishikawa and Kitamura 1999). Further investigation of this interaction could lead to the development of treatments that could exploit these pathways.

These findings also indicate that increased basal ERK 1/2 activity is associated with increased sensitivity to clofarabine. This may have clinical relevance as tumours expressing higher basal ERK activity are associated with more aggressive tumours and reduced survival rates (Gee *et al.* 2001; Zhang *et al.* 2004; Derin *et al.* 2008; Chen *et al.* 2009).

In conclusion, clofarabine was shown to be a potent cytotoxic agent against ER-negative breast cancers. The favourable pharmacodynamics, tolerability by patients and excellent potency of clofarabine dictate potential for the use of this drug clinically. This is important as, although ER-negative tumours generally respond well to cytotoxics, disease reoccurrence is high and is associated with high mortality (Carey *et al.* 2007). Clofarabine has the potential to be effective as a single agent or in combination therapy and could add a new class of drug to treat aggressive breast cancers.

7.2 Future Perspectives

Solid tumours are known to be hypoxic due to delayed or reduced angiogenesis and stimulation of angiogenesis feeds the tumour and allows it to grow (Blay et al. 1997; Ryzhov et al. 2007). Therefore, the role of adenosine receptors in promoting angiogenesis could be investigated *in vitro* as this has been suggested as a function of adenosine receptors (Blay *et al.* 1997; Linden 2005; Merighi *et al.* 2007; Ryzhov *et al.* 2007). ELISAs could be used to look at the expression of angiogenesis promoting factors such as interleukin-8 (IL-8), fibroblast growth factor (FGF) and VEGF (Auchampach 2007) in the presence of adenosine and selective adenosine receptor agonists and antagonists.

Clofarabine shows the most clinical potential as demonstrated by its pharmacodynamics (Parker *et al.* 1991; Zhenchuk *et al.* 2009) and *in vitro* activity here. Extending the cytotoxicity/apoptosis studies into other breast cancer cell lines would help verify the data presented here. Extending these studies to *in vivo* models would also be necessary. Although clofarabine showed equal potency between ER-negative and leukaemia cells *in vitro*, the solid tumour microenvironment means that drugs often cannot reach all the cells of the tumour, especially those deep within the tumour that are not well vascularised (Minchinton and Tannock 2006). Limited studies have shown, however, that clofarabine did have good (nM) activity against a range of solid tumour xenograft models *in vivo* (Takahashi *et al.* 1999; Waud *et al.* 2000).

Further experimentation is needed to fully understand the mechanisms of action and sensitivity to clofarabine. These studies may then provide insight into which tumours are sensitive to clofarabine treatment. As primary phosphorylation by dCK is not rate-limiting (Gandhi *et al.* 2003) investigating the expression and enzyme kinetics of NMPKs and NDPKs would determine if these kinases are rate-limiting, and if they have a role in the sensitivity to clofarabine.

Further work is needed to characterise the exact role cN-II plays in resistance to NAs. This would require determining clofarabine mono-, di- and triphosphate metabolism by methods such as HPLC (Parker *et al.* 1991; Lotfi *et al.* 1999). Gene knockdown studies using methods such as siRNA would also be useful to determine if expression of cN-II mediates clofarabine sensitivity.

Determining the exact role of ribonucleotide reductase in the activity and sensitivity of clofarabine in breast cancer cells would also be important. The results of this thesis showed that clofarabine was most likely working via inhibition of this enzyme and that increased resistance to clofarabine was correlated with increased p53R2 expression. Therefore studies could be carried out to first identify the inhibition of ribonucleotide reductase by clofarabine and subsequent physiological changes such as dNTP alterations. This could be looked at by HPLC to measure dNTP levels and also by looking at incorporation of clofarabine into the DNA using radiolabelled clofarabine (Månsson *et al.* 1999). Secondly, to determine the role of p53R2 in clofarabine sensitivity, siRNA studies would be useful.

Finally, future studies should characterise the different pathways of induction of apoptosis by clofarabine in different breast cell lines. Chapter 6 demonstrated that the ERK 1/2 pathway was important in the activity of clofarabine in MDAMB231 but not MCF-7 cells. Understanding how ERK 1/2 is important in transducing the apoptotic signal may offer new pathways to target. Clofarabine has been shown to act directly upon the mitochondria causing release of cytochrome c, APAF-1 and caspase 9 (Genini *et al.* 2000a), which could be studied using Western blotting or ELISA to quantify the accumulation of these inducers. This could be furthered by looking at accumulation of important molecules of the intrinsic apoptosis pathway such as pro-apoptotic (Bax, Bad and Bid) and anti-apoptotic (Bcl-2) proteins.

7.3 Thesis Summary

In summation, the following conclusions are drawn from results presented in this thesis:

- MCF-7 and MDAMB231 cells express all four adenosine receptors, but these receptors may not be viable targets for breast cancer treatment.
- The endogenous compounds adenosine and 2'-deoxyadenosine can reduce cell viability by mechanisms requiring intracellular phosphorylation, and adenosine receptor activation to a lesser extent.
- Clofarabine has excellent *in vitro* activity against ER-negative breast cancer comparable to its activity in a leukaemic cell line.
- The activity of clofarabine is dependent upon phosphorylation by dCK and it acts upon ribonucleotide reductase.
- In ER-negative MDAMB231 cells, the action of clofarabine is partly dependent upon ERK 1/2 signalling, but clofarabine does not effect ERK 1/2 phosphorylation directly.
- Increased resistance to clofarabine in the ER-positive cell lines is likely to be due to a combination of factors that include increased levels of the metabolising enzyme cN-II and the DNA repair enzyme, p53R2.
- Clofarabine has therapeutic potential against ER-negative and TNBC and further work should be carried out to investigate this potential.

Chapter 8: References

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