Identification of compounds that may be repurposed for the treatment of triple negative breast cancer

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By

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ABSTRACT

Triple negative breast cancers (TNBC), lack the estrogen and progesterone receptors as well as the HER2 receptor. Currently, there is no selective therapy for the treatment of TNBC and current treatment regimens rely on a cytotoxic chemotherapeutic approach. In addition, many TNBC's display markers of clinically relevant drug resistance, which contribute to the difficulty of treating the disease. Thus, there is a need for the development of new drugs. Current drug discovery is a labourious, costly and time-consuming process with a high attrition rate. Repurposing is an attractive alternative, as many clinically approved compounds have activities unrelated to their current application, that can be harnessed for the treatment of an alternative clinical indication. Here, the Johns Hopkins Clinical Compound Library (JHCCL), consisting of ~1,500 clinically approved and off-patent drugs, was screened against the multi-drug resistant (MDR)-TNBC cell line MDA16. The aim was to identify compounds that caused collateral sensitivity or compounds that displayed novel and/or effective activities against the TNBC cell lines. Three related antimetabolite drugs cytarabine, gemcitabine and cladribine were identified to cause collateral sensitivity. However, collateral sensitivity has been well documented with this class of drug and because of this lack of novelty, their activities were not further characterised. The antibiotic anisomycin (ANS) was identified as the most effective compound from the secondary screen. ANS was found to be highly effective against a subset of TNBC cell lines and against some prostate cancer cell lines *in vitro*. In the MDA-MB-468 TNBC cell line, ANS was shown to induce ribotoxic stress, as evidenced by JNK activation, with induction of caspase-3 like activity and caspase-3 processing as reported in the literature. However, induction of ribotoxic stress and caspase activity is not required for cell death as blocking ribotoxic stress signalling and caspase activity failed to protect cells from ANS induced cell death. These results are contrary to the published literature relating to the mode of cell death in response to ANS treatment. Thus, these findings are novel and increase the repurposing potential of ANS for the treatment of TNBC.

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"Keep away from people who try to belittle your ambitions. Small people always do that, but the really great make you feel that you, too, can become great" - Mark Twain

Dedicated to my family (including Michelle and Basil)!

Abbreviations

ABC	ATP binding cassette transporter family
ANS	Anisomycin
Bcl-2	B-cell lymphoma-2
BSA	Bovine serum albumin
Caspase	Cysteine-dependent aspartic acid specific protease
CHX	Cycloheximide
CLAP	Chymotrypsin, Leupeptin, Antipain, Pepstatin-A
CPX	Ciclopirox
CTL	Cytotoxic T lymphocyte
dCk	Deoxycytidine kinase
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethylene glycol-bis(2aminoethyl)-N,N,N',N'-tetraacetic acid
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Flourescein-5-isothiocyanate
HBSS	Hanks balanced salt solution
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HTS	High-throughput screening
JHCCL	Johns Hopkins Clinical Compound Library
LAPTM4b	Lysosomal associated protein transmembrane-4b
MCL-1	Myeloid cell leukemia sequence-1
MDR	Multi-drug resistant
MPA	Mycophenolic acid
NCE	New chemical entity
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PDK1	Phosphoinositide dependent kinase-1

PR	Progesterone receptor
PTEN	Phosphate and tensin homologue deleted on chromosome 10
ROS	Reactive oxygen species
RPM	Revolutions per minute
SCLC	Small cell lung carcinoma
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SEM	Standard error of the mean
SERM	Selective oestrogen receptor modulator
STS	Staurosporine
TBS	Tris buffer saline
TEMED	N,N,N',N'tetramethylethylenediamine
TNBC	Triple negative breast cancer
VEDF	Vascular endothelial growth factor

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Chapter 1
General Introduction

1.1 Triple negative breast cancer

Triple negative breast cancer (TNBC), is a heterogenous disease accounting for approximately 15% of all breast cancer diagnoses. Clinically, TNBC's are classified by immunohistochemistry as tumours that lack the steroid hormone receptors - estrogen receptor (ER) and progesterone receptor (PR), as well as the human epidermal growth factor receptor-2 (HER2) (Foulkes et al., 2010). Presently, there are no selective therapies for the treatment of TNBC and current treatment regimens rely on cytotoxic chemotherapy. Additionally, many TNBC's display markers of multi-drug resistance (MDR), contributing to the difficulty in treating this particular disease (De Laurentiis et al., 2010) and illustrates that there is a need for newer and more effective drugs. To further exacerbate this problem, the current drug discovery process is protracted, costly and is associated with a high attrition rate. Thus, in recent years alternative approaches for drug discovery have been sought and one approach that seems attractive is drug repurposing. Drug repurposing refers to the identification of a new therapeutic potential for a drug clinically approved for a different disease. This approach is attractive as a wealth of preclinical, clinical and post marketing data exists outlining the pharmacological and toxicological profile of the drug (Ashburn and Thor, 2004).

In addition to TNBC's, a subset of basal-like breast cancer (BLBC) are also negative for ER, PR and HER2 receptors as determined by immunohistochemistry. However, BLBC has recently been classified using gene expression profiling of an intrinsic 500 gene set and is reportedly a more homogenous disease compared to TNBC (Bertucci et al., 2008, Sorlie et al., 2001). At present there is no definitive approach to easily distinguish between TNBC and BLBC in the clinic and as a result the terms TNBC and BLBC have been used interchangeably. What both subtypes have in common is for the majority of BLBC's and TNBC's, there are currently no targeted therapies. A small proportion of TNBC and BLBC possess a mutation in both the *BRCA 1* and *BRCA2* genes, which play key roles in genome stability, detecting and repairing single and double strand breaks (Ashworth, 2008). Mutation of the *BRCA* genes has been shown to increase the dependency of cancer cells to an alternative repair mechanism, poly (ADP-ribose) polymerase (PARP) (Fong et al., 2009). This dependency is exploited by targeting the PARP repair pathway using PARP inhibitors, preventing the cancer cells ability to repair DNA strand breaks and thereby inducing synthetic lethality.

Thus, inactivation mutation of *BRCA* alone is insufficient to induce cell death, but inhibition of PARP in combination with inactivated *BRCA* induces synthetic lethality in cancer cell lines *in vitro* (Ashworth, 2008). PARP inhibitors have also been shown to possess anticancer activities in patients with *BRCA* mutated tumours (Fong et al., 2009).

Due to the lack of effective and targeted therapies for the treatment of TNBC, current treatment regimens consist of aggressive cytotoxic chemotherapeutics. TNBC respond most favourably to a combination of anthracycline and taxane based chemotherapeutics, such as doxorubicin and taxol as well as to regimens containing 5-fluorouracil (5-FU) and cyclophosphamide (Rouzier et al., 2005). Initially, response rates to the cytotoxic chemotherapeutic regimen are highly favourable, which is most likely attributed to the rapid rate of proliferation of the tumour cells (De Laurentiis et al., 2010). However, relapse with the development of drug resistance to current chemotherapeutic regimens is a major problem (De Laurentiis et al., 2010, Arslan et al., 2009, Santana-Davila and Perez, 2010). These factors contribute significantly to the difficulty in effectively treating this breast cancer subtype. In addition to lacking targetable receptors, some non-*BRCA* mutated TNBCs also possess mutant p53, elevated cyclin-E and epidermal growth factor receptor (EGFR), high ki-67 index and low cyclin-D levels (Umemura et al., 2005). Furthermore, many TNBC's express proteins known to be associated with MDR, such as amplification of lysosome associate protein transmembrane 4-b (LAPTM4-b) and YWHAZ gene which encode for the anti-apoptotic scaffold protein 14-3-3ζ (Li et al., 2010b). Taking this into consideration there is an evident need for more potent and selective therapies for the treatment of TNBC.

1.2 Multi-drug resistance in breast cancer

The development of drug resistance, whether intrinsic or acquired is a major factor limiting the effective treatment of breast cancer. MDR occurs when tumours become unresponsive to multiple drugs that are both structurally dissimilar and have different mechanisms of action. MDR can occur through a number of different mechanisms as outlined in (Figure 1.1). These mechanisms have been extensively studied *in vitro* and subsequently validated *in vivo* and include amplification of efflux proteins such as MDR1, which decreases cellular accumulation of cytotoxic drugs including the mainstay of breast cancer chemotherapy doxorubicin, mutation or deletion of tumour suppressor genes such as phosphate and tensin homologue deleted on chromosome 10 (PTEN) and p53 (O'Connor et al., 2007).

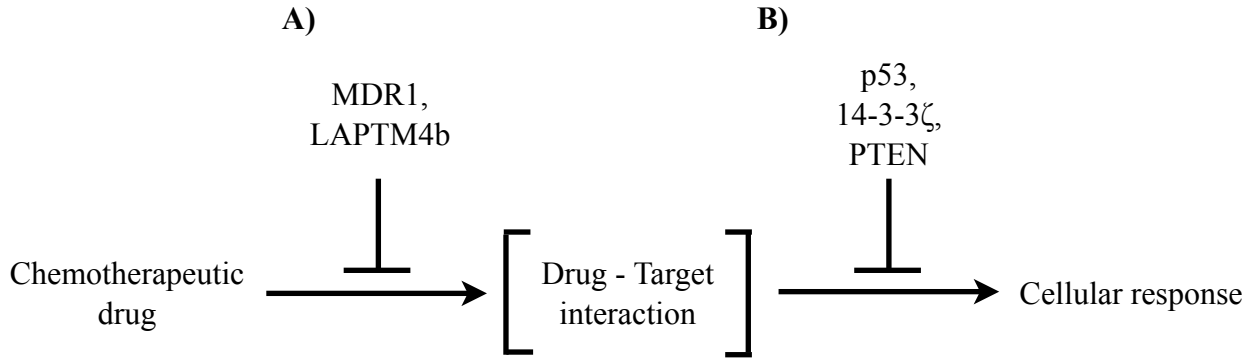


Figure 1.1: Mechanisms of clinically relevant drug resistance - The mechanism of drug resistance can occur by preventing the interaction between drug and target or by perturbation of the subsequent downstream signalling events in response to the drug. **A)** Resistance can be conferred by amplification of efflux pumps that prevent the cytotoxic chemotherapeutic agent from reaching its intended cellular target. **B)** Following interaction of a cytotoxic chemotherapeutic with its target, resistance can develop by altered signalling downstream of the target and thereby prevent induction of the cell death response or indeed mutations in the drug target can result in resistance.

Below are a number of clinically relevant genes and their protein products that confer MDR in breast cancer patients and some have been identified to possess MDR in other cancer types. Table 1 outlines these genes as well as clarifying their reported cellular function under normal circumstances. Development or identification of compounds that can reverse or overcome the resistance induced by amplification of these genes of over-expression of their proteins is essential for the treatment of patients with MDR breast cancer.

Gene	**Protein**	**Normal function**
P53	p53	Cell cycle and apoptosis regulator (Hanahan and Weinberg, 2011)
PTEN	PTEN	Phosphatase that negatively regulates the Akt signalling pathway (Dahia, 2000)
ABCB1	MDR1	Transport of cellular metabolites (Sharom, 2008)
YWHAZ	14-3-3-zeta	Scaffold protein (Sunayama et al., 2005)
LAPTM4B	LAPTM4B	Transport of nucleoside analogues (Hogue et al., 1996)

Table 1 - Normal function of genes known to contribute to multi-drug resistance in breast cancer: Over-expression or amplification of these genes or their protein products have been shown to contribute to clinically relevant multi-drug resistance.

1.2.1 p53

The p53 gene encodes for the p53 protein and is one of the best characterised tumour suppressors. p53 plays a central role in a number of critical cellular pathways, inducing the expression of genes involved in cell cycle arrest, senescence, apoptosis, autophagy and regulation of metabolism (Hanahan and Weinberg, 2011, Brady and Attardi, 2010). However, p53 can also repress gene expression by sequestering co-repressors to target gene binding domains, additionally, p53 can repress gene expression by activation of microRNAs (Brady and Attardi, 2010). Breast cancer patients expressing elevated levels of p53 immunohistochemical staining, have reduced survival compared to patients with low p53 staining (Alsner et al., 2008), furthermore, approximately 50% of all breast cancers have mutated p53, which is associated with poor prognosis (Aas et al., 1996, Wilson et al., 1997). Most p53 mutations occur in the DNA binding domain, thereby perturbing p53's ability to induce target gene transcription, thus preventing p53 from eliciting its tumour suppressive effect (Brady and Attardi, 2010). Missense mutations in the p53 DNA binding domain are associated with worse prognosis compared to missense mutations lying outside structural/ conserved domains (Alsner et al., 2008). Interestingly, some of these mutations can confer gain of function capabilities on p53 and have been associated with increased invasiveness and metastasis (Lang et al., 2004). Apart from its nuclear effect, cytoplasmic p53 plays a role in directly regulating apoptosis. Following induction of an appropriate stress response, p53 levels increase and promote mitochondrial outer membrane permeabilisation (MOMP) (Brady and Attardi, 2010). It is believed that this occurs, either by p53 binding to the anti-apoptotic proteins Bcl-2/Bcl-X_L, allowing for the pro-apoptotic proteins Bax and Bak to form pores in the mitochondria or by p53 directly binding to Bax, releasing it from the anti-apoptotic protein MCL-1 (Leu et al., 2004). These protein-protein interactions are known to rely on the DNA binding domain of p53 (Leu et al., 2004). Thus, cancers with p53 mutations residing in the DNA binding domain are not capable of inducing p53 dependent mitochondrial apoptosis.

Chemotherapeutics such as the DNA damaging agents doxorubicin and cyclophosphamide and the microtubule poison vincristine, can activate p53 (Wilson et al., 1997). Active p53, induces the expression of genes that play roles in cell cycle arrest and DNA repair pathways, in an attempt to repair drug induced DNA damage. If the cell is irreparably damaged, p53 induces the expression of genes associated with mitochondrial or intrinsic apoptosis as well as directly interacting with anti-apoptotic proteins, resulting in cell death (Brady and Attardi, 2010).

Thus, tumours expressing mutant p53 are associated with MDR to a range of structurally different and mechanistically diverse compounds compared to tumours over-expressing wild type p53 (Aas et al., 1996, Wilson et al., 1997, Longley and Johnston, 2005).

1.2.2 PTEN

PTEN protein is a phosphatase that dephosphorylates phosphatidylinositol (3, 4, 5) triphosphate, a second messenger which activates the Akt pro-survival signalling pathway. PTEN thus, acts as a negative regulator of the Akt signalling pathway (Dahia, 2000). Deletion or mutations of the tumour suppressor PTEN or amplification in downstream effectors such as Akt, is associated with endometrial, prostate and 40-50% of breast cancers (Depowski et al., 2001). Loss of PTEN expression is associated with an aggressive disease and there is a significant correlation between loss of PTEN and lymph node metastasis as well as disease related death (Depowski et al., 2001). Small studies testing the effect of PTEN expression in primary human tumours have reported that PTEN deficient HER2 over-expressing breast cancers have significantly reduced response rates to trastuzumab compared to HER2 over-expressing PTEN wild-type tumours (Fujita et al., 2006, Nagata et al., 2004). Loss of PTEN and the subsequent development of resistance to trastuzumab has been illustrated both *in vitro* using breast cancer cell lines and *in vivo* using breast cancer xenograft models. From this data, it was believed that trastuzumab's mechanism of action was solely reliant on PTEN expression and treatment with trastuzumab increased the activity of PTEN, thereby reducing Akt signalling, resulting in decreased cell survival (Nagata et al., 2004). However, it has since been discovered that trastuzumab elicits an antitumour effect by inducing a cytotoxic t-cell immune response via its ability to coat the outside of the cancer cell, signalling for its destruction (Mimura et al., 2005, Barok et al., 2007).

Loss of PTEN is also associated with development of resistance to both doxorubicin and tamoxifen in breast cancer cell lines and to gefitinib in lung cancer cell lines *in vitro* (Steelman et al., 2008, Zhuang et al., 2009). Development of resistance to both doxorubicin and tamoxifen is believed to result from amplified Akt signalling due to loss of PTEN. It has been shown *in vitro* that PTEN deficient breast cancer cell lines are highly sensitive to inhibitors of mammalian target of rapamycin (mTOR), a downstream effector of Akt.

Introduction of wild type PTEN into these PTEN null breast cancer cell lines, restores sensitivity to doxorubicin and conversely results in insensitivity to the mTOR inhibitor rapamycin (Steelman et al., 2008). Low levels of PTEN in the lung cancer cell line H-157 was associated with resistance to gefitinib. However, irradiation induced PTEN expression, sensitised the H-157 lung cancer cell line to gefitinib (Zhuang et al., 2009). Thus, these data illustrate the contribution that loss of PTEN confers in development of MDR in a range of different cancers. Targeting downstream effector proteins such as mTOR inhibitors in the treatment of PTEN null breast cancers may offer an exploitable therapeutic intervention.

1.2.3 The ATP binding cassette (ABC) transporter gene family

The ABC transporter protein superfamily are involved in the transport of a wide variety of xenobiotics including anti-cancer drugs, lipids and metabolic proteins from the intracellular environment (Sharom, 2008). Some of the most well characterised ABC family members include ABCB1 (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP1). *In vitro,* the over-expression these ABC family members is associated with resistance in breast (MDR1, BCRP1), prostate and lung (MRP1) cancer cell lines to anthracyclines and alkaloids, chemotherapeutics that are both structurally dissimilar and mechanistically diverse (Leonard et al., 2003, Mechetner et al., 1998). Over-expression of MDR1 or P-glycoprotein and its role in conferring MDR is extremely well documented *in vitro*, however, due to conflicting reports its relevance to drug resistance in human tumours is considered controversial (Trock et al., 1997). However, in recent years a number of factors have been identified that can explain the inconsistency relating to the detection of MDR1 and lack of a clinical effect using MDR1 inhibitors. The sensitivity of the methods used to detect protein expression has improved dramatically over the last two decades. Early methods of detection relied on RNA-hybridization based assays, which have poor sensitivity and do not allow for the discrimination between malignant versus stromal tissue or the presence of infiltrating blood cells. In addition, RNA-hybridisation does not reflect protein levels and as such can yield negative conclusions regarding the potential role of MDR1 in drug resistant breast cancers (Trock et al., 1997). The development of immunohistochemistry based assays for the detection of protein levels has become the gold standard and is much more robust than RNA hybridisation. Most importantly, immunohistochemistry allows for the discrimination between malignant and stromal tissues.

A study comparing the detection ability of MDR1 expression using RNA-hybridisation and immunohistochemistry reported that MDR1 was detectable in less than 27% of breast tumours using RNA-hybridization compared to approximately 50% with immunohistochemistry (Trock et al., 1997). Poor experimental design contributed to some of the reports that discredited the role of MDR1 in conferring drug resistance. These included MDR1 expression levels being measured prior to administration of chemotherapy instead of measuring MDR1 expression levels before and after chemotherapy and the use of cytotoxic chemotherapeutics that are not MDR1 substrates being included in studies attempting to ascertain a role of MDR1 in resistance (Trock et al., 1997). To further illustrate the correlation between MDR1 and drug resistance, results from a meta-analysis study reported that MDR1 expression increased significantly in the tumours of patients that received cytotoxic chemotherapeutics that are known MDR1 substrates. Additionally, patients with tumours over-expressing MDR1 are three to four times more likely to be unresponsive to chemotherapy and therapeutic intervention may increase MDR1 expression potentially leading to the development of acquired resistance (Trock et al., 1997). It has been reported, that approximately 30% of tumours previously treated with MDR1 related chemotherapeutics have a three fold increase in MDR1 expression compared to tumours not previously treated with cytotoxic agents (Mechetner et al., 1998). MDR1 over-expression correlated with increased resistance of primary tumour cells *in vitro* to doxorubicin (approximately 1.5 fold) and taxol (approximately 2.5 fold). No change was observed in response to 5-FU, a non-MDR1 substrate (Mechetner et al., 1998). These data suggest that MDR1 is a valid marker of MDR with significant clinical relevance.

The potential of compounds that specifically interfere with MDR1 activity being used in combination with cytotoxic agents has been tested to determine if resistance can be reversed. Unfortunately, MDR1 inhibitors have had limited success in clinical trials due to lack of tumour response. Factors that contributed to the limited effect of MDR1 inhibitors include; lack of confirmation that MDR1 was over-expressed in patient tumours, lack of evidence of MDR1 inhibition *in vivo* and toxicity when MDR1 inhibitors and chemotherapeutics are used in combination (Leonard et al., 2003). A number of trials that did confirm MDR1 over-expression in tumours, including advanced or metastatic paclitaxel-refractory breast, acute myeloid leukemia and lung cancer have identified a beneficial effect when using first generation MDR1 inhibitors such as cyclosporine A and verapamil (Saeki et al., 2007).

Despite a beneficial effect observed in phase I and II clinical trials, phase III clinical trials were associated with high failure rates due to dose limiting toxicity or reducing the dose of chemotherapeutics below levels required for beneficial effect. A small clinical trial testing the effects of the second generation MDR1 inhibitor biricodar in combination with doxorubicin induced 2 partial remissions and 7 stable disease states against paclitaxel refractory breast cancer (Bramwell et al., 2002). Recently, the third generation MDR1 inhibitor dofequidar fumarate, used in combination with cyclophosphamide, doxorubicin and 5-FU in breast cancer patients was associated with an increased response rate (complete and partial response) of 25%, compared to patients that did not receive dofequidar fumarate. The inhibitor also increased progression free survival by approximately four months and was well tolerated (Saeki et al., 2007). Thus, second and third generation MDR1 inhibitors are less toxic than first generation inhibitors and are associated with greater response rates.

An imaging technique has been developed to demonstrate functional inhibition of MDR1 by MDR1 inhibitors *in vivo*. This approach utilises the radionucleotide Technetium-99m sestamibi, which is a known MDR1 substrate (Ciarmiello, 1998). Imaging has been performed to determine the rate of sestamibi efflux from tumours and it has been suggested that a rapid sestamibi efflux from the tumour can predict potential lack of tumour response to chemotherapeutics which are known MDR1 substrates (Ciarmiello, 1998). A phase I clinical trial containing patients with metastatic drug resistant cancers known to over-express MDR1 were co-treated with the MDR1 inhibitor tariquidar and sestamibi. Tariquidar treatment resulted in accumulation of sestamibi in tumours indicating the potential use of sestamibi to assess functional MDR1 inhibition in patient tumours *in vivo* (Agrawal et al., 2003).

To conclude, MDR1 over-expression is associated with clinically relevant drug resistance and much of the confusion that has arisen can be associated with poorly designed experiments and techniques which lacked the sensitivity required to detect functional MDR1. Therefore, future trials need to implement a number of criteria in order to obtain the most accurate results. Stratification of patients based on MDR1 over-expression in tumours, confirmation that MDR1 plays a central role in resistance, the choice of inhibitor, the anticancer drug regimen employed and the use of sestamibi or an alternative method for determining the degree of functional MDR1 inhibition.

1.3 Drug resistance in TNBC

Development of drug resistance to doxorubicin, using TNBC cell lines has been illustrated *in vitro*. Amplification of two genes, *YWHAZ* and *LAPTM4b*, have been illustrated to confer this resistance to doxorubicin. In addition, amplification of these genes is observed in breast cancer patients whose tumours have relapsed and are resistant to anthracycline base chemotherapy **(Li et al., 2010b)**.

1.3.1 *YWHAZ*

The YWHAZ gene encodes for the anti-apoptotic scaffold protein 14-3-3ζ. The 14-3-3 protein family are well known scaffold proteins, that bind to phosphorylated serine resides on target proteins preventing their ability to interact with downstream effecters and thereby inhibit induction of cell death (Tsuruta et al., 2004, Neal et al., 2011). Amplification of the YWHAZ gene and subsequent over-expression of its protein 14-3-3ζ, has been shown to contribute to clinically relevant MDR in patients with large B-cell lymphoma and breast cancer (Danes et al., 2008, Li et al., 2010b). Amplification of the YWHAZ gene in breast cancer patients is reportedly associated with a more aggressive disease phenotype (Li et al., 2010b). 14-3-3ζ regulates apoptosis by binding to phosphorylated ser112 and ser136 on the pro-apoptotic protein Bad but also targets other pro-apoptotic proteins including Bax and Foxo3a (Sunayama et al., 2005, Tsuruta et al., 2004). Following stimulation of a stress response, 14-3-3ζ itself is antagonised by up-stream pro-apoptotic stress sensor proteins which phosphorylate 14-3-3ζ on serine residue 184 thereby preventing its ability to bind and sequester target proteins. The stress activated kinase c-Jun NH_2-terminal kinase (JNK) is a known antagoniser of 14-3-3ζ activity. In response to genotoxic and ribotoxic stress JNK is activated resulting in 14-3-3ζ phosphorylation on serine-184. This phosphorylation event releases Bad and Bax which translocate to the mitochondria subsequently releasing cytochrome c and inducing apoptosis (Sunayama et al., 2005). JNK is reported to phosphorylate Bad directly on serine-128, this death associated phosphorylation event reduces the affinity of Bad for 14-3-3ζ (Sunayama et al., 2005). Over-expression of 14-3-3ζ *in vitro* using the non-cancer breast cell line MCF10a induced malignant transformation, evident by increased anchorage-independent growth, luminal filling and down-regulation of p53 (Danes et al., 2008). YWHAZ is amplified in the TNBC cell line MDA-MB-231, conferring resistance to doxorubicin and silencing YWHAZ using siRNA has been shown to reverse resistance to doxorubicin (Li et al., 2010b).

Additionally, a retrospective study has reported that 14-3-3ζ over-expression is significantly associated with increased activation of the Akt survival pathway. This association correlates with significantly reduced disease free survival in breast cancer patients (Neal et al., 2011). *In vitro,* 14-3-3ζ has been shown to bind and localise phosphatidyl-inositol 3 kinase (PI3K) to the plasma membrane allowing for PI3K to interact with phosphoinositide-dependent kinase 1 (PDK1), increasing PI3K activity and subsequently increasing the activity of the downstream effecter Akt (Neal et al., 2011). Taken together, these data illustrate that amplification of the YWHAZ is associated with clinically relevant drug resistance and *in vitro,* amplification of YWHAZ in non-cancer cell lines can induce malignant transformation. Interestingly, the YWHAZ and LAPTM4B genes are found on the chromosomal locus 8q22, which is part of an amplicon consisting of twelve genes. It is likely that amplification of the chromosomal locus 8q22 confers resistance by the interaction of both 14-3-3ζ and LAPTM4b (Li et al., 2010b). The remaining ten genes in the amplicon were shown not to confer resistance *in vitro*. Amplification of LAPTM4b prevents doxorubicin from reaching its cellular target, the nucleus and amplification of 14-3-3ζ antagonises the cells ability to induce that apoptotic pathway. This illustrates the complexity of drug resistance and the need to identify drugs that can overcome resistance and circumvent the inability of the cell to undergo apoptosis possibly by inducing another form of cell death such as autophagy.

1.3.2 LAPTM4b

LAPTM4b is located on chromosome 8q22 and encodes a 35kDa transmembrane glycoprotein pump expressed on endosomal and lysosomal membranes in yeast and human cell lines *in vitro*. LAPTMs are known to be involved in the transportation and sub-cellular localisation of nucleosides and a range of xenobiotic agents, including a number of anticancer compounds across plasma membranes (Vergarajauregui et al., 2011, Hogue et al., 1996). Transport of nucleosides across the plasma membrane is an important process as it allows for salvaging of extracellular nucleosides that can be reused for energy metabolism or nucleic acid synthesis. The chromosomal locus 8q22, is amplified in some breast cancers and associated with amplification of the LAPTM4b gene. Over-expression of LAPTM4b in breast cancer patients is associated with decreased disease free survival and poorer clinical outcome in response to adjuvant chemotherapy (Li et al., 2010b). The TNBC cell line MDA-MB-231 over-expresses LAPTM4b, which has been shown to confer resistance to the anthracycline doxorubicin (Li et al., 2010b).

LAPTM4b has been shown to confer resistance in the MDA-MB-231 cell line by sequestering doxorubicin in lysosomal membranes, thereby preventing the cytotoxic threshold of doxorubicin being achieved in the nucleus. siRNA mediated knockdown of LAPTM4b was associated with a significant increase in phosphorylated-H2AX staining. This indicates the induction of DNA damage and suggests that a cytotoxic level of doxorubicin was achieved in the nucleus following ablation of LAPTM4b (Li et al., 2010a). A potential explanation for the resistance and poorer prognosis associated with amplified LAPTM4b, is that doxorubicin accumulation in the nucleus does not reach a cytotoxic threshold and the induction of cell death is prevented. LAPTM4b is also up-regulated in a number of other solid tumours including hepatocellular, lung and ovarian cancers and this is associated with poor prognosis (Kasper et al., 2005). Determining the exact compounds that LAPTM4b amplification confers resistance to, is important for ensuring patients whose tumours have amplified LAPTM4b do not receive a combination of drugs that contain LAPTM4b substrates. Testing a range of currently available therapies against clinically relevant markers of drug resistance, such as MDR1 and LAPTM4b, may in the short term offer a solution for identifying compounds that possess anticancer activities against MDR cancers.

Testing the activity of potentially novel anticancer agents *in vitro* requires the use of cancer cell lines. The cell lines used in this thesis are reported to express some of the aforementioned markers of clinically relevant drug resistance and are thus, the most appropriate *in vitro* model for identifying and characterising potentially novel anticancer agents. For a list of the cancer cell lines and their particular mutations see Appendix 5. Discovery or identification of compounds that can overcome MDR and subsequently kill cancer cells is important for future therapies. TNBC's display some or all of the above markers of MDR which contributes significantly to the difficulty in effectively treating this form of breast cancer.

1.4 Drug Discovery

Novel drug discovery is an expensive and labour intensive process that currently has an extremely low success rate despite pharmaceutical companies investing billions in research, development and marketing. *De novo* drug discovery is a massively protracted process. It has been estimated that, from the initial identification of a compound, determining its toxicological and pharmacological activity *in vitro*, using preclinical models and in clinical trials can take 15-20 years before the compound is approved and marketed. Additionally, taking a drug from the concept stage to its final clinical indication, can cost between $500 million and $1 billion depending on the complexity of the compound and the manufacturing process (Adams and Brantner, 2006). Despite such intensive investment of time, effort and resources the successful approval of a drug is not guaranteed. In 2011, thirty four new chemical entities (NCE) were approved, the highest number recorded in recent years (FDA, 2011). Despite this small increase in the amount of approved NCE the number still pales in comparison to that of failed drugs. This illustrates that there is a problem with the current approaches to drug discovery as the number of successfully approved drugs is significantly less than the number of drugs which have failed.

1.4.1 Target based drug discovery

Conventional anticancer chemotherapeutics such as DNA damaging agents; doxorubicin and etoposide demonstrate a degree of selectivity by effectively killing rapidly proliferating cells. This lack of discrimination between rapidly proliferating healthy and cancerous cells gives rise to some of the most common side effects associated with DNA damaging agents such as nausea and immune suppression. Rational anticancer drug discovery is aimed at tailoring drugs that target a single molecule believed to be the "Achilles heel" of the cancer and thus being the "magic bullet" that cures the patient of the cancer, while simultaneously reducing unwanted side effects commonly associated with conventional cytotoxic drugs. This method of drug discovery has resulted in the manufacturing of highly target specific compounds such as the monoclonal antibodies; trastuzumab and bevacizumab as well as the specific kinase inhibitor imatinib. However, these rationally designed drugs did not, in the majority of instances, cure the disease and one of the most interesting findings was that they did possess off-target effects. It is now known that less than 35% of breast cancers over-expressing HER2 respond to trastuzumab therapy and patients that initially respond rapidly develop resistance (Liberato et al., 2007).

The most significant off-target effect induced by trastuzumab is cardio-toxicity (Keefe, 2002). This side effect is further exacerbated when trastuzumab is administered in combination with doxorubicin and can result in dose limiting cardio-toxicity (Keefe, 2002). However, not all side effects are deleterious. Imatinib is a targeted kinase inhibitor, that was manufactured to specifically inhibit the constitutively active BCR-ABL fusion protein which contributes to aberrant signaling in chronic myeloid leukemia. Imatinib has subsequently been shown to bind to and inhibit platelet derived growth factor receptor (PDGF-R) and c-kit (Shah et al., 2002). These off-target effects have proven beneficial as imatinib has been shown to possess anticancer activities against gastrointestinal stromal tumours via inhibition of PDGF-R and c-kit.

Development of resistance to targeted therapies such as trastuzumab, imatinib and bevacizumab has been reported in patients with breast cancer (Miller et al., 2007, Shah et al., 2002). Considering the cost of manufacturing targeted therapies, the development of resistance is a major hurdle that faces all future targeted therapies. Targeted therapies have the benefit of being highly selective with a subset of patients that respond well to their treatment, however, the targeting of a single protein can also be a disadvantage. Despite some patients responding favourably, the development of targeted therapies that are specific for a particular cellular molecule, believed to be the key mediator in a multifactorial disease has as yet, proven unsuccessful at inducing complete remission in cancer patients. One of the most obvious examples, is the failure of trastuzumab to induce complete remission in patients with HER2 over-expressing breast cancers (Vogel et al., 2002). The initial lack of response and subsequent development of resistance to targeted therapies such as trastuzumab and bevacizumab represent a significant limitation for single-target based drug discovery. Indeed, despite the investment required to manufacture targeted therapeutics, clinically they are rarely administered as a single agent due to limited therapeutic effectiveness and onset of resistance. In fact, trastuzumab and bevacizumab are administered in combination with current cytotoxic chemotherapeutics such as doxorubicin or oxaliplatin respectively. The use of combination therapy aims to target major cellular pathways such as DNA damage (doxorubicin or etoposide), or microtubule stabilisation (paclitaxel). Thus, a poly-pharmacological approach aimed at perturbing several molecular factors that results in tumour cell death is currently being employed. This combination approach, which currently is the most effective therapy, is associated with dose limiting toxicity and contraindications. An alternative drug designing approach is the development of a poly-pharmacological drug i.e. a drug with multiple cellular targets.

Indeed, this idea has been suggested in an attempt to increase the effectiveness and specificity of drugs for the treatment of central nervous system disorders, which are known to be polygenic in nature such as depression and schizophrenia (Roth et al., 2004). Clozapine is the current gold standard treatment of schizophrenia and is the epitome of a "dirty" drug, as it interferes with multiple cellular targets. Using a reductionist based approach, researchers hypothesised and subsequently developed targeted therapies that interfered with receptors believed to be the key mediators of schizophrenia, with the preconception that these compounds would be more efficacious and associated with less adverse side effects (Tuunainen et al., 2002). However, in the clinic these compounds were found to be less effective than clozapine and were associated with significant adverse side effects (Roth et al., 2004, Tuunainen et al., 2002). Interpretation of these findings and relating them to cancer therapy, suggests that future drug development should aim to identify and produce pharmaceutical compounds that are not "magic bullets", but instead "magic buckshot" designed to interfere with several key factors essential for cancer cell survival. This "magic buckshot" or "carpet bombing" approach has taken the interest of researchers in the last number of years with many looking at the structures of currently approved drugs as a scaffold for multi-targeting drugs (Pujol et al., 2009). It seems that the concept of multi-targeting drug is well developed at present with numerous research groups proposing that applying a computational, network based approaching as the best method to identify these compounds (Csermely, 2007, Csermely et al., 2005, Hopkins, 2008, Jia et al., 2009). However, as yet there has been no reports of pharmaceutical companies developing multi-targeting drugs. Despite this, one could foresee that within the next decade there will be an emergence of multi-targeted drugs, not only for cancer but for other diseases that are poly-genetic in nature.

An alternative approach to rational drug discovery is high throughput screening (HTS) to identify compounds that kill cancer cells *in vitro*. This approach unlike rational drug discovery requires significantly less pathophysiological knowledge of the cancer. Similar to rational drug discovery however, is the cost and time required to develop that compound from the concept stage to clinical trials and approval. This is an extremely protracted process that can take over a decade and cost up to $1 billion dollars (Adams and Brantner, 2006). A related approach is to screen compounds that are currently approved for the treatment of other diseases to determine if these compounds possess anticancer activities. This process is known as drug "repurposing" or "repositioning".

One of the major advantages of drug repurposing, is that it requires little pathophysiological knowledge and significantly less time for approval due to the wealth of preclinical and clinical information relating to it toxicity and pharmacological activity. Additionally, in theory, these compounds should cost significantly less to develop and approve compared to rationally designed drugs.

1.4.2 Repurposing

In an attempt to reduce both the time and cost of bringing a compound from concept to the clinic, more innovative and efficient methods of drug discovery are being sought. A recent area of interest is repurposing of FDA approved drugs for new clinical indications (Ashburn and Thor, 2004). One of the major advantages of repurposing compounds is that a wealth of pharmacokinetic, pharmacodynamic and toxicological data has already been amassed. Additionally, in theory, these compounds are relatively easy to manufacture, produce and have already been in the market for decades. As such, the the cost of the therapy to the patient should be a fraction of the cost currently paid for "designer" target therapies. It has been estimated that putting an approved drug through clinical trial for a new purpose can take as little as two years and cost approximately $10-50 million, 10-20 times less than that of a NCE (Hareyan, 2007). In principle, this represents enormous savings but more importantly, the accelerated development translates to better patient care, faster. Libraries of such FDA approved drugs are commercially available, such as the John Hopkins Clinical Compound Library (JHCCL) and have been screened extensively *in vitro* against a large number of disease models. To date, only two FDA approved drugs, thalidomide and the SERM raloxifene, have been repurposed as anticancer therapies (Sukhai et al., 2011). However, a growing number of compounds are being identified that possess cytotoxic properties *in vitro* and using preclinical models *in vivo* some of which may be advanced into clinical trials

1.3.3 Successfully repurposed compounds

Over the last ten years only eight known drug compounds were repurposed for the treatment of various cancers and of these six were known anticancer agents (Sukhai et al., 2011). The remaining two compounds thalidomide and raloxifene, were not known anticancer agents. Thalidomide was used for the treatment of morning sickness in the 1940s and 1950s and was subsequently withdrawn from the market following the identification that is was a potent teratogen.

However, it was repurposed initially for the treatment of erythema nodosum laprosum (ENL), a painful inflammatory condition associated with leprosy. In was not until the late 1990's that thalidomide was found to possess potent anticancer activities against refractory multiple myeloma, which at the time had no effective treatment (Teo et al., 2002, Singhal et al., 1999). Raloxifene is a second generation SERM, related to the SERM tamoxifen and was initially prescribed for the treatment of osteoporosis. Raloxifene was successfully repurposed for the treatment of HR positive breast cancer in 2007 and was found to decrease the incidence of breast cancer in women at a high risk of developing the disease (Bevers, 2006). Unlike tamoxifen, raloxifene is not associated with an increased risk of developing endometrial cancer, cataracts, thrombosis or osteoporosis. The exact mechanism owing to the reduction of these side effects is not as yet fully understood (Vogel et al., 2006). Therefore addition of raloxifene to the current treatment regimen allows for protection of the bone marrow thus, preventing development of osteoporosis as well as exerting its own anticancer activity. Raloxifene has been shown to possess potent anticancer activities *in vitro* against breast (Okamoto et al., 2008, Stuart and Rosengren, 2008), prostate (Kim et al., 2002a, Kim et al., 2002b) and myeloma (Olivier et al., 2006) cancer cell lines.

1.4.3 Drugs in clinical and preclinical testing

There has been significant interest in repurposing over the last five years which has led to the identification of numerous compounds that possess novel cytotoxic activities *in vitro* and some have even shown promise in preclinical xenograft models. Indeed, HTS of clinically approved drug libraries has resulted in the identification of compounds that elicit highly cytotoxic activities against cancer cell lines *in vitro*. Two examples of compounds identified to possess cytotoxic activities against cancer cell lines by HTS using clinically approved drug libraries are the antifungal ciclopirox olamine (CPX) and the antibiotic nitroxoline.

CPX was recently identified from two independent screens testing the potential cytotoxic properties associated with off-patent, non-neoplastic drugs against a panel of haematological cancer cell lines. This *in vitro* cytotoxic activity was confirmed in preclinical haematological cancer xenograft models *(Eberhard et al., 2009)*. Due to these findings a clinical trial has been established testing the potential anticancer activities of CPX against seven haematological malignancies (http://clinicaltrials.gov/ct2/show/NCT00990587).

Nitroxoline was recently identified from a screen to possess cytotoxic activities *in vitro* against breast cancer and bladder cancer cell lines and subsequently *in vivo*, using preclinical xenograft models of breast and bladder cancers *(Shim et al., 2010)*.

Thus, these compounds have been shown to elicit cytotoxic activities *in vitro* using cancer cell lines and this has been validated in preclinical cancer xenograft models *in vivo*. Repurposing of FDA approved compounds that possess anticancer activity can help to overcome the problems associated with both rationally designed targeted therapies and novel drug discovery. As previously discussed a poly-pharmacological approach for the treatment of a multi-mutation based disease such as cancer, seems like the most logical approach for the development of future therapies. Following the successful repurposing of a compound, it is likely that in a clinical setting the most beneficial anticancer effect will be achieved by combining targeted and cytotoxic therapies. Repurposing of compounds identified as possessing cytotoxic activities *in vitro* and subsequent validation of effectiveness in clinical trials will increase the currently depleting pool of anticancer agents available on the market. This comes with the benefit that, in theory, these compounds should be cheaper to buy than the designer labeled targeted therapies. Indeed there are already more potent analogues of thalidomide, lenalidomide, which has recently been approved for the treatment of refractory multiple myeloma. Other analogues are being tested against solid tumours including prostate cancers, gliomas and melanomas where potent anticancer effects have been observed in phase II and III clinical trials *(Aragon-Ching et al., 2007)*. Thus, using drugs that have been shown to possess anticancer activity as a template for designing of more potent and safe anticancer compounds in the future, may be the way forward to winning the war against breast cancer.

1.5 Aims

TNBC, unlike other breast cancer subtypes, currently does not have any targeted therapies. In the clinic, treatment of TNBC relies on an aggressive cytotoxic chemotherapeutic approach. While most TNBC respond favourably to cytotoxic chemotherapy, some TNBC are inherently insensitive and in those that initially respond, development of resistance can occur rapidly. Thus, there is a need for the identification and development of more selective and efficacious drugs for the treatment of TNBC. However, development of novel rationally designed anticancer agents is a protracted process with a significantly high failure rate. In an attempt to speed up this process "repurposing" of currently approved drugs has recently become an attractive alternative. Indeed, to date two compounds have been successfully repurposed, one compound is currently in clinical trials and large number of compounds have been illustrated to possess cytotoxic activities against cancer cell lines *in vitro* and in preclinical cancer xenograft models thus, suggesting their potential as novel anticancer agents. Taken together this illustrates the potential of repurposing as a viable option for profiling the activities of currently approved drugs against different diseases.

The aims of this thesis were to:

i) Screen the Johns Hopkins Clinical Compound Library (JHCCL) against the MDR-TNBC cell line MDA16

ii) Validate hits from JHCCL screen using MDA16 and the parental MDA-MB-468 cell line to identify compounds that caused collateral sensitivity and compounds that possess effective or novel activities against these cancer cell lines

iii) Characterise the activity of selected compounds

Chapter 2
Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

MCF-7, BT-474, SkBr3, MDA-MB-231, DU-145, HCT-8, HT-29 and SW480 cancer cell lines were obtained from the American tissue culture collection (ATCC) (Rockville, MD). MDA-MB-468 and MDA16 breast cancer cell lines were a kind gift from by Dr. Timothy Grant (Medical Research Council (MRC), toxicology unit, University of Leicester). The non transformed breast cell line MCF10a and the breast cancer cell line T47D were kind gifts from Dr. Roisin Dwyer (Clinical Science Institute (CSI)-Galway). Hs578t, HCC1937 and BT20 breast cancer cell lines were a kind gift from Dr. Lorraine O'Driscoll (Trinity College Dublin (TCD)). 22Rv1 and PC3 prostate cancer cell lines were a kind gift from Dr. Sharon Glynn (National Centre for Biomedical Engineering Science (NCBES)).

2.1.2 *Culture conditions*

MCF7, BT-474, SkBr3, MDA-MB-468, MDA16, MDA-MB-231, HCT-8, HT-29, SW480 and DU145 cancer cell lines were maintained in DMEM medium supplemented with 10% FBS, 100 U/ ml penicillin and 100 µg/ml streptomycin. T47D, Hs578t, HCC1937 and BT20 breast cancer cell lines were maintained in RPMI medium supplemented with 10% FBS, 1% L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. 22Rv1 and PC3 prostate cancer cell lines were maintained in F-12 Hams medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. MCF10a were maintained in F-12 Hams medium supplemented with 5% Horse serum, 20 ng/ml epidermal growth factor (EGF)-long, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone. Cell lines were incubated in a humidified incubator (5% CO_2, 37°C) and routinely passaged every 2-3 days.

2.1.3 Freeze-down of cell lines

Freeze-down of cell lines was performed by resuspending trypsinised cells in medium. Cells were maintained at a cell density of 2 x 10^6 cells/ml. Supplemented medium containing 20% DMSO was added to this cell suspension drop-wise giving a final cell density of 1 x 10^6 cell/ml and a final DMSO concentration of 10%. 1ml aliquots of this cell suspension were added to cryogenic vials. Cells were gradually frozen to reduce cell death, cryogenic vials were loaded into a polystyrene holder and placed into a -70°C freezer. After 48 hours storage in a -70°C freezer, cryogenic vials were transferred into liquid nitrogen for long term storage.

Cell lines were reconstituted by rapidly thawing cryogenic vials in a 37°C water-bath. Cells were transferred to sterile 15 ml tubes and 5 ml of supplemented medium was added. Cells were spun-down at 300xg, for 5 minutes. Cell pellets were resuspended in 5 ml supplemented medium and transferred to a T25 flask and were incubated at 37°C with 5% CO_2.

2.2 Analysis of cell viability and apoptosis

2.2.1 *Alamar blue assay*

Alamar blue is a fluorescent dye that is a substrate of cellular reductase enzymes. In viable cells, cellular reductases reduce resazurin to highly fluorescent resourfin (O'Brien et al., 2000). Readouts from this assay are commonly reported as cell viability, however, this is misleading as the assay cannot discriminate between cells that have arrested, become senescent or if a proportion of the cell population have died. Due to this limitation, the readout of the alamar blue assay in this study was explicitly interpreted as a measurement of cellular reductase activity.

Cells were seeded in a 96-well plate, at a cell density of 10,000-30,000 (depending on the cell line) and were allowed to adhere overnight. Drug concentration responses were performed using the concentration range (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100μM). Cell lines were incubation in the drug for either 24 or 48 hours, alamar blue was added to cells at a final concentration of 112μM and cells were incubated in alamar blue for 6 hours at 37°C. After incubating cells in alamar blue for 6 hours, fluorescence was measured using the Victor 3 plate reader and drug induced changes in cellular reductase activity were calculated.

2.2.2 *Colony formation assay*

Cells were seeded at a density of 1,000 cells per well in a 6 well plate and incubated overnight at 37°C, 5% CO_2. Cells were subsequently treated with control or drug for 0, 2, 8, 12, 24 and 48 hours. At each time-point the drug was washed away using supplemented medium and fresh medium was added. Cells were allowed to grow for one week replacing fresh medium every three days. After one week colonies were washed using ice cold PBS and fixed in methanol for 5-10 minutes. Following fixation, crystal violet dye was added and incubated with the cells for 5-10 minutes. Crystal violet was washed away using cold PBS and colonies were left to dry overnight.

Colonies were visualised by light microscopy and the number of colonies per well was determined by scoring colonies with greater than five cells.

2.2.3 *Caspase-3 like activity assay*

Cells were harvested by adding 1ml of ice cold phosphate buffered saline (PBS), into a T25 flask and scraping cells into 1ml PBS. Cells are transferred to labelled 1.5ml eppendorf tubes. Cells were pelleted by centrifugation using a bench-top centrifuge at 4°C for 5 minutes at 300xg. Supernatant was removed and cell pellet was resuspended in 60µl of lysis buffer keeping cells on ice. 56µl of lysed samples were transferred to 96-well flat bottom, black coated assay plates (CoStar) followed by 144 µl of substrate buffer. The fluorogenic caspase-3 tetrapeptide substrate Ac-DEVD-AFC fluoresces when active caspase-3 cleaves the amide bond releasing the AFC fluorophore. The degree of fluorescence is determined by the concentration of free AFC and was measured using a Cytofluor 4000 fluorometer (Perceptive Diagnostics), at excitation wavelength, 400nm and emission wavelength, 508nm. Caspase-3 like activity was normalised to protein concentration determined by the Bradford assay and activity was expressed as arbitrary fluorescent units per minute (AFU) per mg protein.

*Buffers and reagents: (*See appendix 1)

2.2.4 *YO-PRO assay*

Adherent cells were lifted using 1ml 0.2% EDTA which was neutralised by adding 5 ml FACS buffer (980mL PBS, 20mL FBS and 0.5g Sodium Azide). Cells were pelleted by centrifugation at 300xg for 5 minutes. Cell pellets were resuspended in 1ml FACS buffer and transferred to FACS tubes. Samples were separated into unstained, YO-PRO single stained and propidium iodide (PI) single stained. Cells were incubated at 37°C for 15 minutes in YO-PRO. FACS tubes containing cells were washed with FACS buffer and cells were centrifuged at 300xg for 5 minutes to decrease non-specific fluorescence. A final wash was performed by resuspending the cell pellet in 5ml FACS buffer and cells were centrifuged at 300xg for 5 minutes and the supernatant was removed. Cell pellets were resuspended in 1ml FACS buffer. Unstained samples were used to set voltages to determine the baseline fluorescent intensity of the cells.

50 µg/ml PI was added to detect the necrotic cell population. Using PI and YO-PRO as a double stain the level of necrosis and apoptosis was measured using a scatter plot. Stained cells were analysed using the Flowjo software.

2.2.5 *TMRE assay for mitochondrial membrane depolarization (Δψm) and fluorescence microscopy*

The MDA-MB-468 cell line was seeded ($1x10^6$ cells in a $25cm^2$ dish) and left to adhere overnight. MDA-MB-468 cells were subsequently treated with 1µM ANS for 24 hours. MDA-MB-468 cells were treated with the positive control, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCCP), at a concentration of 50µM for 4 hours. Tetramethylrhodamine ethyl ester perchlorate (TMRE) a dye that accumulates in mitochondria with polarised mitochondrial membranes, was added to the culture medium to give a final concentration 100 nM and the cells incubated at 37^oC for 30 minutes. In some experiments MDA-MB-468 cells were stained with Hocehst 33342 (1µg/ml) for 10 minutes to stain DNA. MDA-MB-468 cells stained with Hocehst 33342 and TMRE were visualised using a fluorescent microscope. MDA-MB-468 cells treated with CCCP and TMRE were harvested by trypsinisation and resuspended in warm HBSS and were subsequently transferred to a 96 well plate (200µl per well). Fluorescence was measured using the Accuri flow cytometer.

2.2.6 Transfection of the MDA-MB-468 cell with siRNA for MCL-1

The MDA-MB-468 cell line at a cell concentration of $5x10^5$ cells were reverse transfected with 100nM of either control, FITC-siRNA or MCL-1 siRNA using Neo FX siPORT. MDA-MB-468 cells were incubated in each siRNA for 48 hours. After this time, FITC-siRNA was used to determine the transfection efficiency using the Accuri flow cytometer. Both control and MCL-1 siRNA treated MDA-MB-468 cells were incubated in 112µM alamar blue for six hours at 37°C, 5% CO_2. Fluorescence was read at 560nm using the Wallac Victor 3 1420 Multilabel counter (PerkinElmer).

2.3 Preparation and screening of the Johns Hopkins Clinical Compound Library (JHCCL)

2.3.1 Formatting of the JHCCL and primary screening

The MDR-TNBC cell line MDA16 was trypsinised, counted and resuspended in DMEM medium yielding a final cell density of 150,000 cells per ml. The JANUS liquid handling robot (PerkinElmer) seeded MDA16 cells at a cell density of 30,000 cells per well. Once seeded cells were allowed to adhere by incubating at 37°C, 5% CO_2 overnight. JHCCL drug plates were obtained in 96 well plate format at a drug concentration of 10mM. The JANUS liquid handling robot performed a series of drug dilutions in order to yield a final drug concentration in the cells of 10μM.

i) A 1:10 dilution of JHCCL was made into a 96 well plate containing DMSO yielding a drug concentration of 1mM in 100% DMSO.

ii) A 1:10 dilution of the above plate was made into a fresh 96 well plate containing DMEM yielding a drug concentration of 100μM and 10% DMSO.

iii) The final drug dilution was made 1:10 into the MDA16 cell plate yielding a final drug concentration of 10μM and 1% DMSO.

Once MDA16 cells were treated with the JHCCL, cells containing the drug were incubated at 37°C, 5% CO_2 for 48 hours. After this time the alamar blue assay was performed by incubating cells in 112μM alamar blue for 6 hours and subsequently reading fluorescence at 560nm using Wallac Victor 3 1420 Multilabel counter (PerkinElmer).

2.3.2 *Secondary screening of hit compounds identified from the JHCCL*

A 10mM working stock of each drug was made, aliquoted and stored at -20°C for future use. The TNBC cell line MDA468 and the MDR-TNBC cell line MDA16 were trypsinised and a cell count was performed. After counting, cells were seeded at a density of 30,000 cells per well and incubated at 37°C, 5% CO_2 overnight. Serial dilutions of each drug was made using DMEM medium (1% DMSO) yielding a dose response of each compound (0, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100μM). All medium was removed from cell plates and replaced with the appropriate drug concentration. Following the addition of each drug, cells were incubated for 48 hours at 37°C, 5% CO_2.

After the 48 hour incubation in drug, 112μM alamar blue was added, incubated with cells for 6 hours at 37°C, 5% CO_2 and fluorescence was read at 560nm using the Wallac Victor 3 1420 Multilabel counter (PerkinElmer).

2.4 Protein detection

2.4.1 Bradford assay

Cells were harvested as outlined above and cell pellets were resuspended in lysis buffer. Samples were incubated on ice for 5 minutes. A standard curve was established using known concentrations of BSA to determine the protein concentration of samples. 2μl of each sample was aliquoted in duplicate into a 96 well flat bottom plate and 198μl of bradford reagent was added to the sample yielding a final volume of 200 μl per well. Absorbance was measured at 595nm using a Wallac Victor 3 420 Multilabel counter (PerkinElmer) allowing protein concentration to be determined.

2.4.2 Immunoblotting

Materials and reagents: (See appendix 4)

Pelleted cells were lysed on ice for 5-10 minutes using a suitable quantity of lysis buffer. Protein concentration was determined using the bradford assay. Lysed samples were denatured in SDS-laemmli buffer and heated to 95°C for 5 minutes. Equal amounts of protein from each sample was resolved using 8-15% SDS-polyacrylamide gels. The percentage of gel selected was dependent on the molecular weight of the protein of interest. Proteins resolved on gels were transfered to PVDF membrane under the following conditions; 25V, 4°C overnight or 100V at room temperature for 1 hour using 1X transfer buffer containing 10% (v/v) methanol. After transferring, membranes were stained with Ponceau S stain (0.1% Ponceau S, 5% acetic acid) to access equal protein loading. Ponceau S was washed off the membrane using either 1X tris buffered saline, tween (TBST) or 1X PBST. Membranes were blocked overnight at 4°C or for 2 hours at room temperature, in 5-10% dried milk in PBS or TBS-0.05% Tween. After blocking the membranes they were incubated with primary antibody at concentrations, either overnight at 4°C or for 1 hour at room temperature on a rocker.

Following incubation in primary antibody, membranes were washed three times in PBS/TBS-Tween 0.05% and incubated in horseradish peroxidase-linked secondary antibody at concentrations detailed below for a minimum of two hours. Protein bands of interest were visualised by adding Immobilon western chemiluminescent HRP substrate according to the manufacturer's instructions. Protein bands were detected and visualised using the Fluorochem chemiluminescent imaging system.

2.4.3 Preparation of samples for detection of phosphorylated proteins

Breast cancer cell lines were treated with ANS for 2, 4 and 6 hours. At each time-point samples were harvested by lysing cells in lysis buffer containing phosphatase inhibitor cocktails to prevent the removal of phosphorylation moieties. 10µl of the lysed samples were taken for protein estimation by the bradford assay. After two minutes, boiled 2X laemmli buffer was added to the lysed sample. Following addition of boiling laemmli buffer, samples were vortexed for 10 seconds and boiled at 95°C for 5 minutes. Immunoblotting was performed as outlined in section 2.4.2.

2.5 Preparation of cells for visualisation by transmission electron microscopy (TEM) and fluorescence microscopy

2.5.1 Preparation of cells for visualisation by TEM

Fixation

After harvesting cells, the cell pellet was resuspended in 3% glutaraldehyde in 0.2M cacodylate buffer for 1-2 hours in 1.5mL plastic eppendorf tubes. Cells were gently spun to get a pellet (400xg for 5 minutes). The cell pellet was washed with 0.1M cacodylate buffer and the resuspended cell pellet was centrifuged (400xg for 5 minutes) x2. Resuspended cell pellet with 1mL of 1% Osmium tetroxide per eppendorf, incubating for 1-2 hours in fume-hood (pelleted cells turn black after osmium step). After fixing in osmium tetroxide, gently spin cells to generate a pellet. Osmium was removed and stored safely for disposal. Ascorbic acid was added to waste osmium to neutralise and cooking oil was added to prevent evaporation. Cell pellet was washed with 0.1M cacodylate buffer. Resuspended cell pellet and centrifuge (400xg for 5 minutes) x2.

Dehydration

From this point on the cell pellet was not resuspended. Solutions were gently pipetted to ensure pellet was not disturbed.

The fixed cell pellet was dehydrated by adding 1mL ethanol (EtOH) in increasing concentrations (30% EtOH for 15 minutes, 50% EtOH for 15 minutes, 70% EtOH for 15 minutes, 95% EtOH for 15 minutes, 100% EtOH for 30 minutes and 100% EtOH for 30 minutes) to each eppendorf. After this process the cell pellet was incubated in a 50:50 EtOH:resin mixture overnight at room temperature.

Embedding

The 50:50 resin was removed and the cell pellet was washed (2 x 2 hour washes using 100% resin to remove residual ethanol present in the pellet). 100% Resin was made fresh and the cell pellet was embedded by adding 0.75mL 100% resin. Samples were left to polymerise for **2-3 days** at 60°C in the fume-hood.

Chapter 3

Screening the Johns Hopkins Clinical Compound Library against the triple negative multi-drug resistant breast cancer cell line MDA16

3.1 Introduction

At present there is no effective targeted therapy available for the treatment of TNBC. Additionally, many TNBCs possess a MDR phenotype contributing to the difficulty in effectively treating this form of breast cancer (Li et al., 2010b). Novel drug discovery is a costly and time-consuming process, that is associated with a high attrition rate. The idea of testing currently approved drugs for potential anticancer activity is known as repurposing and offers an attractive alternative to novel drug discovery. This is due to a wealth of preclinical and clinical knowledge already existing, but most importantly, the reduced time and in principle, the cost of taking a drug from testing to clinical approval.

The aim of this thesis, is to identify drugs from the JHCCL, which consists of approximately 1,500 clinically approved compounds, that possess cytotoxic activities against the MDR1-TNBC cell line, MDA16. Alamar blue is a fluorescent dye that is reduced by cellular reductases in viable cells (O'Brien et al., 2000). The alamar blue assay is a simple one step assay, that is robust and highly reproducible and as such, was selected as the assay to identify “hit” compounds that display effectiveness in decreasing cellular reductase activity from the primary screen. A hit compound was predefined as a compound that decreased cellular reductase activity by greater than three standard deviations from the mean. Hit compounds identified from the primary screen, were subsequently obtained from a source independent from the JHCCL and a secondary screen was performed on the parental MDA-MB-468 and the MDR TNBC sub-line MDA16. This was to validate the findings of the primary screen and to identify compounds that caused collateral sensitivity, or in the absence of collateral sensitivity, compounds that elicited novelty and/or effectiveness at decreasing cellular reductase activity. Compounds that met this criteria, were selected for further characterisation and classification.

However, before the primary screen of the JHCCL was performed, a number of important variables had to be addressed. First and foremost, the MDA16 cell line reportedly possesses a MDR phenotype (Turton et al., 2001) and this phenotype had to be confirmed. Secondly, the alamar blue assay had to be optimised for HTS using the JANUS liquid handling robot and a drug spiking dummy run was performed, to illustrate that this assay was both sensitive and robust enough to detect a hit compound.

3.2 Results

3.2.1 Confirming the phenotype of the MDR-TNBC cell line MDA16

The MDA16 cell line, is a sub-line derived from the parental TNBC cell line MDA-MB-468 and is reportedly MDR owing to over-expression of the transmembrane efflux pump MDR1 (Turton et al., 2001). The MDR phenotype of the MDA16 cell line, was tested by comparing the expression and activity of the MDR1 pump to the parental MDA-MB-468 cell line. The MDA-MB-468 and MDA16 cell lines were seeded at a concentration of $1x10^6$ cells per T25 flask and cells were allowed to adhere overnight. Both cell lines were harvested after 24 hours and samples were prepared for western blotting. Immunoblotting for MDR1 identified that the MDA16 cell line has significant over-expression of MDR1 compared to the parental MDA-MB-468 cell line (Figure 3.1A). Subsequent to identifying that the MDA16 cell line over-expresses MDR1, the activity of the pump was tested using the fluorescent dye Rhodamine 123, a well known MDR1 substrate. The MDA-MB-468 and MDA16 cell lines were seeded at a concentration if $1x10^4$ cells per well and left to adhere overnight. The MDA-MB-468 and MDA16 cell lines were then incubated for one hour in increasing concentrations of Rhodamine 123 (1, 3 and 10μM). After this time medium was removed, cells were washed in HBSS and cells were lysed. The fluorescence associated with lysed MDA-MB-468 and MDA16 cell lines was measured using a spectrophotometer. The MDA-MB-468 cell line exhibited a concentration dependent increase in fluorescence, indicating intracellular accumulation of Rhodamine 123. The MDA16 cell line exhibited a significantly lower level of fluorescence at all concentrations used, indicating a very low intracellular accumulation of Rhodamine 123. This illustrates that the MDA16 cell line over-expresses MDR1, which acts to extrude the substrate Rhodamine 123 from the intracellular environment (Figure 3.1B). The resistance to an MDR1 substrate was tested by performing a 48 hour doxorubicin concentration response on both the MDA-MB-468 and MDA16 cell lines. Doxorubicin is the mainstay chemotherapeutic for the treatment of most breast cancers and is also a well characterised MDR1 substrate. The change in cellular reductase activity in both cell lines, to increasing concentration of doxorubicin, was determined by the alamar blue assay. There was a statistically significant difference between the decreased cellular reductase activity in both the MDA-MB-468 and MDA16 cell lines to doxorubicin. The EC_{50} of doxorubicin in the MDA16 cell line (approximately 30μM), was almost 100 fold greater than the EC_{50} in the MDA-MB-468 cell line (0.3μM), as evidenced by the decrease in cellular reductase activity (Figure 3.1C).

Combined, this data illustrates that the MDA16 TNBC cell line, has over-expressed and increased activity of the MDR1 pump compared to the parental MDA-MB-468 cell line and this correlates with increased resistance to the known cytotoxic doxorubicin, thus correlating with the reported MDR phenotype of the MDA16 cell line.

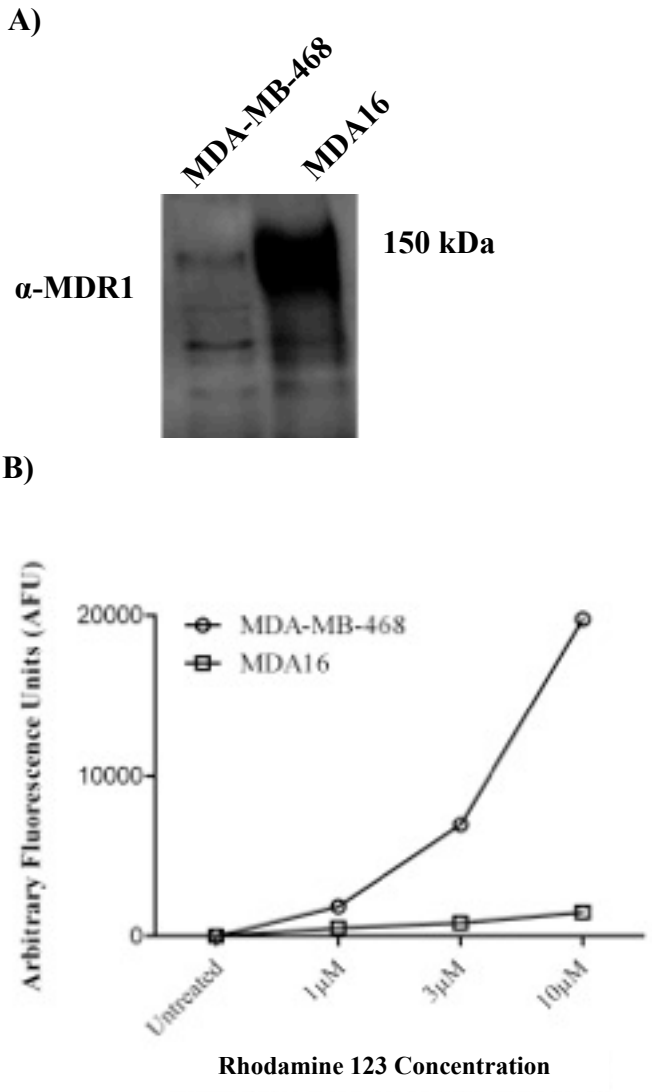


Figure 3.1 The MDA16 cell line possesses a drug resistant phenotype - Expression of MDR1 protein was tested in the breast cancer cell lines MDA-MB-468 and MDA16. **A)** The MDA16 but not parental MDA-MB-468 breast cancer cell line over-expresses MDR1 at the protein level. **B)** Activity of MDR1 was measured using the intracellular fluorescent dye Rhodamine 123, a known MDR1 substrate. MDA-MB-468 and MDA16 cell lines were incubated in Rhodamine 123 for one hour, after this time cells were washed, lysed and fluorescence was measured. The MDA-MB-468 cell line has high levels of Rhodamine 123 fluorescence indicative of high intracellular accumulation of the dye. The MDA16 cell line exhibits low levels of fluorescence indicative of low intracellular accumulation of Rhodamine 123 (Data is representative of three similar experiments). **C)** The MDA16 cell line is significantly less sensitive to the doxorubicin induced decrease in cellular reductase activity compared to the MDA-MB-468 cell line. (Data shown, n=3 ± SEM. Statistically significant differences between the response of MDA-MB-468 and MDA16 cell lines to doxorubicin determined using two way ANOVA followed by Bonferroni post hoc test. **, P<0.01, ****, P<0.0001).

3.2.2 Optimization of Alamar blue assay for detection of hit compounds

Following confirmation that the MDA16 cell line possesses a MDR phenotype, the alamar blue assay was optimised in order to ascertain the most robust criteria required to increase the probability of identifying a "hit" compound(s) through HTS of the JHCCL. The Z-factor is a statistical method for determining robustness and was used during optimisation of the alamar blue assay. A Z-factor of 0.5 - 1 demonstrates a highly robust assay format, conversely a Z-factor < 0.5 indicates a poor assay format (Zhang et al., 1999). During the optimisation procedure, it was identified that systematically controlling a number of key variables generated a large dynamic range, making it easier to identify a hit compound. It was identified that, seeding cells at a concentration of 30,000 cells per well, performing a 48 hour time-course and incubating cells for six hours in 112µM alamar blue yielded the best Z-factor of 0.95 (Figure 3.2). This Z-factor was obtained by manual seeding and drug treatment and thus the next step was to perform a drug spiking experiment, controlling these variables comparing manual and JANUS handled samples.

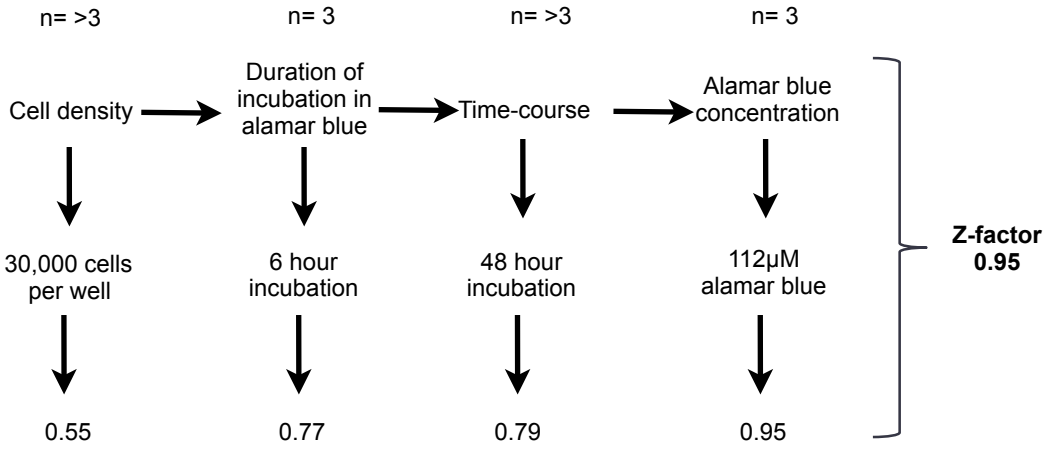


Figure 3.2 Workflow of alamar blue assay optimisation - The above parameters were identified as important variables and were optimised in order to increase the robustness of the screen. Robustness was determined using the Z-factor, which we achieved 0.95. The middle column is the optimal conditions that were controlled and applied for the screen and for all future alamar blue based assays.

96 well plates were seeded with the MDA16 cell line either manually or by JANUS. In parallel, a drug "mother-plate" was established, so that manual and JANUS treated MDA16 cell plates underwent identical treatment regimens. The drug mother-plate was spiked with the cytotoxic agents staurosporine and clotrimazole at a concentration of 10mM in 100% DMSO. Following serial dilutions of the mother-plate, MDA16 cells were spiked with a final drug concentration of 10µM (Figure 3.3A).

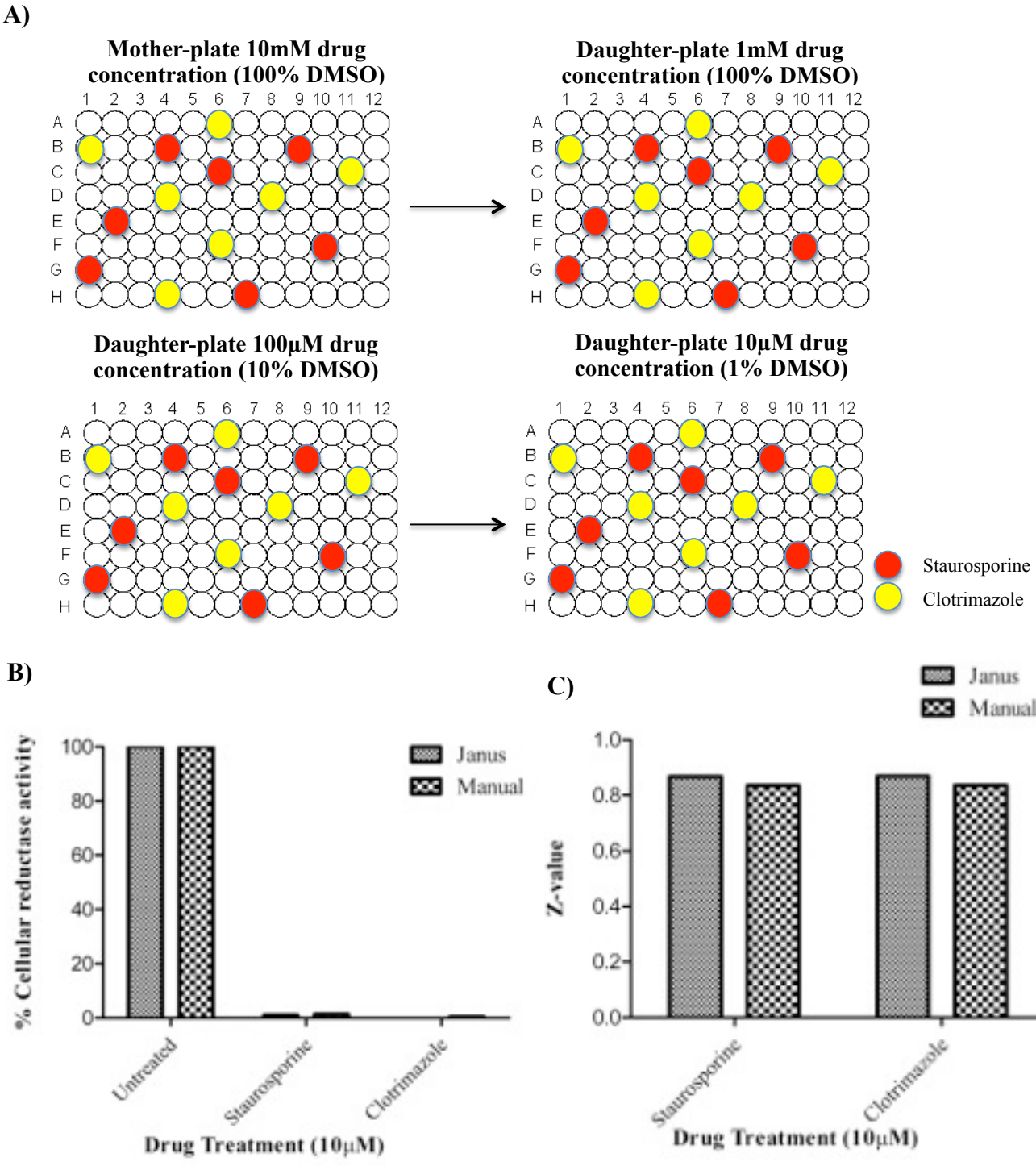


Figure 3.3 Drug spiking the drug resistant TNBC cell line MDA16 and optimisation of the alamar blue assay for HTS - The ability of the alamar blue assay to detect a hit, was tested by performing a blind drug spiking experiment on the MDA16 breast cancer cell line. **A)** A drug mother-plate was generating by randomly spiking wells with staurosporine or clotrimazole at a concentration of 10mM. A first generation daughter-plate was made by making a 1:10 dilution from the mother-plate into DMSO, yielding a drug concentration of 1mM in 100% DMSO. A second generation daughter-plate was made by performing a 1:10 dilution of the first generation daughter-plate into DMEM, yielding a drug concentration of 100µM in 10% DMSO. A final 1:10 dilution was made by adding drugs from the second generation daughter-plate directly into the cell plate, yielding a final drug concentration in the cells of 10µM (1% DMSO). **B)** MDA16 cells were spiked with drugs and incubated for 48 hours, after this time the alamar blue assay was performed. Both clotrimazole and staurosporine induced a significant decrease in cellular reductase activity. **C)** The Z-value of clotrimazole and staurosporine from the drug spiking experiment was calculated and both compounds were associated with a Z-values of ~ 0.8. (results representative of two similar experiments).

Following 48 hour incubation, the alamar blue assay was performed using the previously outlined optimised conditions. Both staurosporine and clotrimazole, which are not MDR1 substrates were highly effective at decreasing cellular reductase activity in the MDA16 cell line (Figure 3.3B) and generated Z-factors > 0.8 irrespective of whether performed manually or using JANUS (Figure 3.3C). Thus, these results illustrate that the alamar blue assay is both sensitive and robust enough to identify "hit" compounds that effectively decrease cellular reductase activity.

3.2.3 Screening the JHCCL for compounds that decrease cellular reductase activity

After confirming the MDR phenotype of the MDA16 cell line and optimisation of the alamar blue assay for the detection of hit compound(s), the JHCCL was screened against the MDA16 cell line. The screen was performed at a concentration of approximately 10μM, a concentration commonly used in HTS. As a rule of thumb, this is the maximal achievable concentration in plasma without serious side effects (Eberhard et al., 2009, Shim et al., 2010).

A "hit" was predefined, as any compound that reduced cellular reductase activity by greater than three standard deviations from the mean, equating to a reduction in cellular reductase activity of approximately 40%. Applying these criteria, resulted in the identification of thirty unique hit compounds, that fall into fifteen different drug classifications (Figure 3.4A, Figure 3.4B). The most represented group were the antineoplastics, consisting of nine hit compounds, five of which were antimetabolite agents (Figure 3.4C). The next most represented class was the dyes and diagnostics containing four compounds; brilliant blue, sodium chromate CR51, tolonidine blue and methylene blue (Figure 3.4D). These compounds are generally administered topically and as such were immediately excluded from further characterisation. The cardiac glycosides group contained four hits, however, there were only three unique cardiac glycosides strophanthin K, acetyldigitoxin and proscillardin (Figure 3.4E). Strophanthin K was represented twice in the JHCCL drug plate. The remaining compounds represented a range of unrelated classes and exhibited varying capabilities at decreasing cellular reductase activity (Figure 3.4F). Only one false positive was identified in the primary screen, a DMSO control well was found to decrease cellular reductase activity to a similar level as some of the most effective hits identified. The next step was to obtain, where possible, the hit compounds identified in the primary screen, from a source independent of the JHCCL to validate the library and the hits identified.

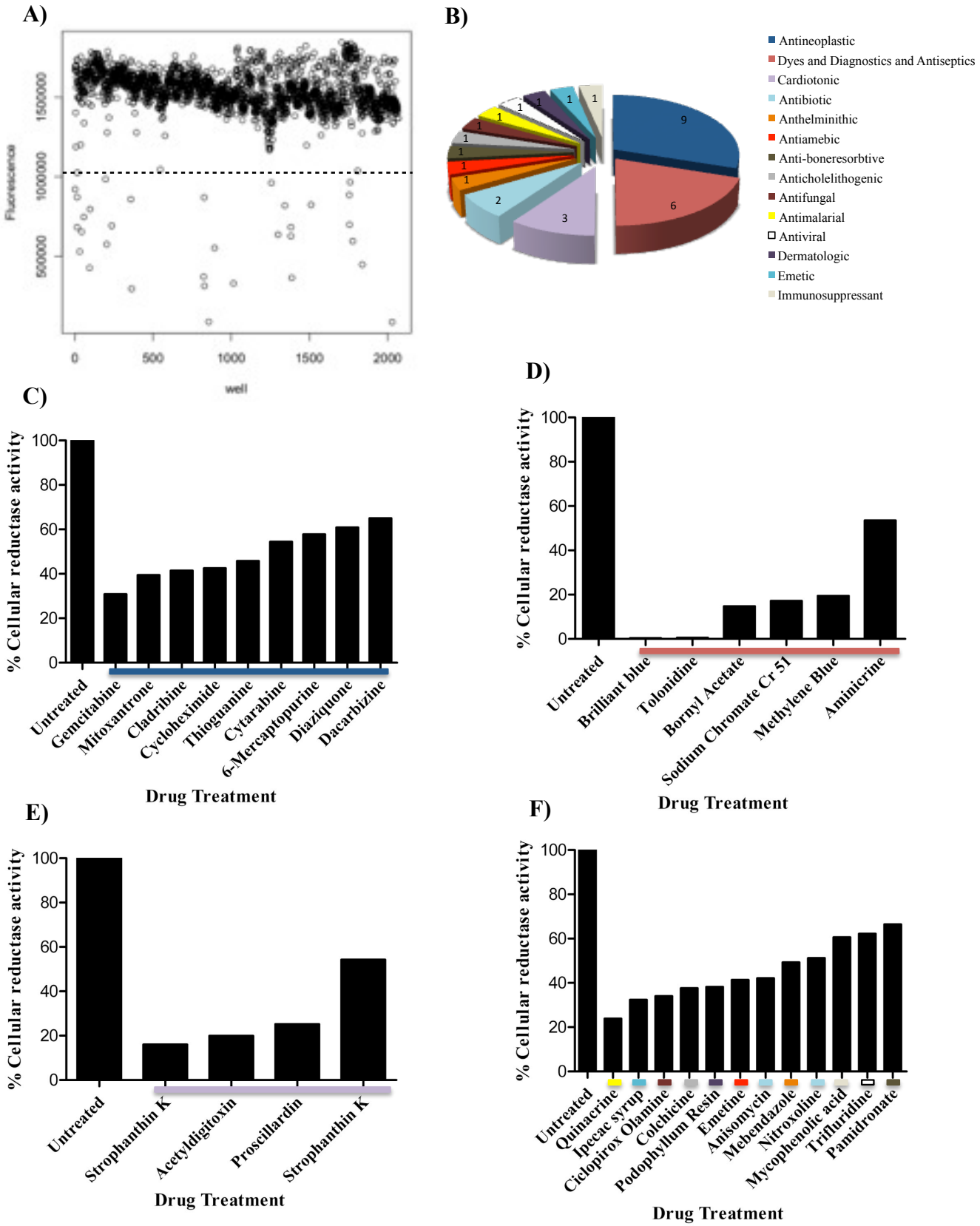


Figure 3.4 Screening the JHCCL and identification of "hit" compounds - The JHCCL was screened against the MDR-TNBC cell line MDA16 at a concentration of 10µM and cells were incubated for 48 hours. After this time-point the alamar assay was performed. **A)** Dot plot of the JHCCL screen, each dot represents a treatment or control. A hit was predefined as a compound that decrease cellular reductase activity by greater than three standard deviations from the mean. Broken line represents the cut off point (Data shown, n=1). **B)** Fifteen different drug classes were identified from the JHCCL screen against the MDA16 cell line. **C)** antineoplastics, **D)** dyes, diagnostics and antiseptics, **E)** cardiac glycosides and **F)** the remaining classes of compounds, identified as hits from the primary screen with varying abilities at decreasing cellular reductase activity Colour coding refers to classifications outlined in figure 3.4b. (Data shown n=1).

3.2.4 Secondary screening of selected compounds independently of the JHCCL

Criteria determined important for selecting hit compounds from the secondary screen (Figure 3.5), were different from the criteria outlined in the primary screen, which was simply to identify cytotoxic agents active against the MDA16 cell line. The secondary screen differed from the primary screen, as both the parental MDA-MB-468 and the MDR-TNBC sub-line MDA16 were treated with the hit compounds. One aim of the secondary screen was identification of compounds that caused collateral sensitivity. Collateral sensitivity refers to the response of a "resistant" cancer cell that has, in the process of becoming resistant to drug-X, becomes hypersensitive to the effects of drug-Y. If a drug was found to cause collateral sensitivity, as evidenced by the drug selectively decreasing cellular reductase activity in the MDR-1 over-expressing MDA16 cell line, while the MDA-MB-468 cell line was significantly less sensitivity, the drug should be novel and effective. In addition, a second aim was to identify a compound that effectively decreased cellular reductase activity in both the MDA-MB-468 and MDA16 TNBC cell lines without necessarily showing collateral sensitivity. This effect was also required to be novel, i.e. no reports that this compound possessed activities against TNBC cell lines *in vitro*. These criteria were considered the most amenable for identification and selection of a compound whose activity is effective and potentially novel.

Compounds identified from the primary screen that were classified as dyes, diagnostics and antiseptics, six compounds in total, were excluded from the list of compounds to obtain as these compounds are generally administered topically. Seven additional compounds were not selected for further characterisation due to their lack of novelty or effectiveness (Table 2).

The cardiac glycosides strophanthin K, acetyldigitoxin and proscillardin and the emetic drug emetine, have previously been reported to possess cytotoxic activities against cancer cell lines *in vitro*. Diaziquone, an antineoplastic has well characterised anticancer activities against a range of human cancers and is associated with significant adverse effects. Both the dermatologic, podophyllin resin and the emetic, ipecac syrup contain a cocktail of active compounds, making the identification of the compound responsible for decreasing cellular reductase activity very difficult. As a result all seven of these compounds were not selected for further validation and characterisation in the secondary screen (Table 2).

The remaining seventeen compounds from the original thirty identified in the primary screen, were obtained from Sigma Aldrich (Table 3) and the secondary screen was performed on the MDR-TNBC cell line MDA16 and the parental MDA-MB-468 cell line..

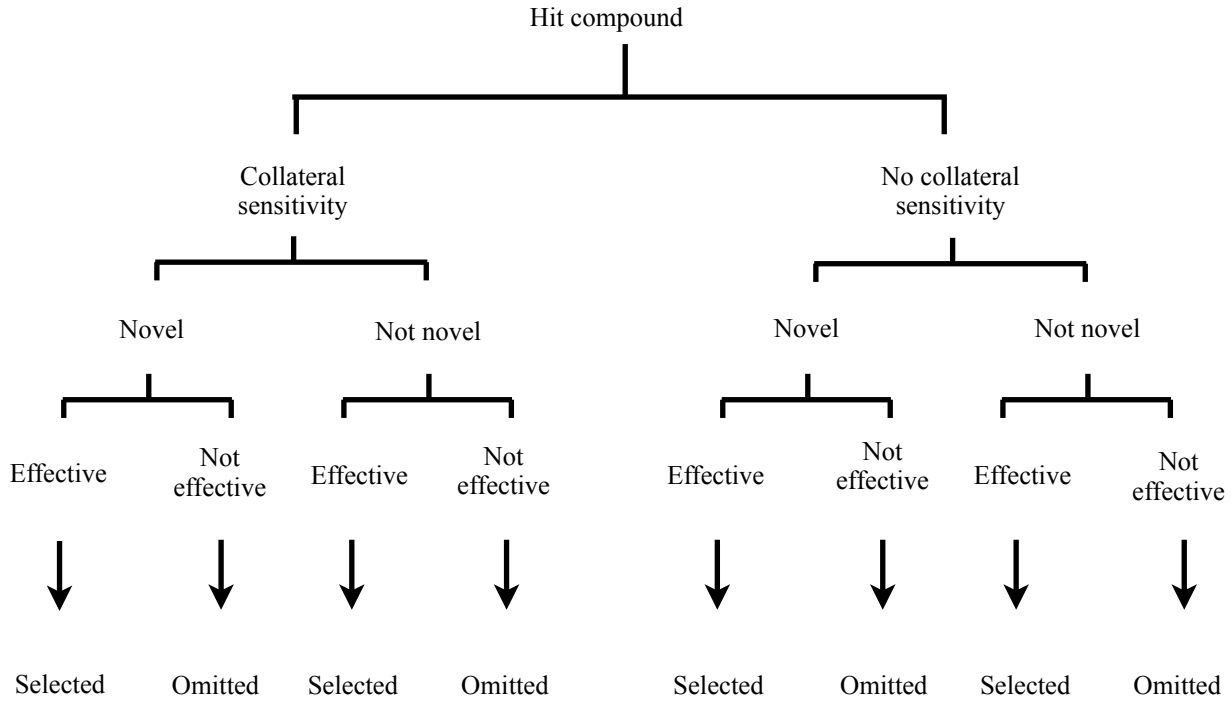


Figure 3.5 - Decision tree format outlined for the selection of a hit compound validated in the secondary screen - Prior to performing the secondary screen, a decision tree based approach was outlined in order to identify and select a candidate compound. The ability of a compound to induce collateral sensitivity was the ultimate aim, however, the effect had to be effective or novel. In the event that no compound met this criteria, it was outlined that a compound would also be selected if the activity was found to be either effective or novel but ideally both.

Compound	Classification	Mode of action
Brilliant Blue	Dye	n/a
Sodium Chromate	Dye	n/a
Methylene Blue	Dye	NAD(P)H:quinone oxidoreductase
Tolonium Chloride	Dye	NAD(P)H:quinone oxidoreductase
Borynl Acetate	Antiseptic	n/a
Aminacrine	Antiseptic	Interacts with DNA
Strophanthin K	Cardiac glycoside	Inhibits Na/K ATPase pumps
Proscillardin	Cardiac glycoside	Inhibits Na/K ATPase pumps
Acetyldigotoxin	Cardiac glycoside	Inhibits Na/K ATPase pumps
Diaziaquone	Antineoplastic	Alkylating agent
Podophyllin Resin	Dermatologic	n/a
Ipecac syrup	Emetic	Activates chemoreceptive trigger zone
Emetine	Emetic	Protein synthesis inhibitor

Table 2 - List of compounds omitted from further characterised - Thirteen compounds identified from the primary screen, that were omitted from the secondary screen. These compounds were omitted due to their classification, lack of novelty or effectiveness in the primary screen

Compound	Classification	Mode of action
Thioguanine	Antineoplastic	Inhibition of DNA and RNA synthesis
Cytarabine	Antineoplastic	Inhibits DNA synthesis
Gemcitabine	Antineoplastic	Inhibits DNA synthesis
Cladribine	Antineoplastic	Inhibits DNA synthesis
Cycloheximide	Antineoplastic	Protein synthesis inhibitor
Dacarbizine	Antineoplastic	Alkylating agent
Mitoxantrone	Antineoplastic	Inhibits DNA synthesis
6-Mercaptopurine	Antineoplastic	Inhibition of DNA and RNA synthesis
Trifluorodine	Antiviral	Inhibits DNA synthesis
Anisomycin	Antibiotic	Protein synthesis inhibitor
Nitroxoline	Antibiotic	Methionine aminopeptidase-2
Ciclopirox	Antifungal	Iron chelation
Mebendazole	Antihelminthic	Microtubule poison
Colchicine	Anticholeolithogenic	Microtubule poison
Pamidronate	Anti-bone resorptive	Absorbs calcium phosphate
Mycophenolic acid	Immunosuppressant	Inhibits purine biosynthesis
Quinacrine	Antimalarial	Inhibits NF-kB signalling

Table 3 - Seventeen compounds obtained for the secondary screen - The seventeen compounds were screened at a concentration of 10μM against the MDR-TNBC cell line MDA16 and the parental MDA-MB-468 cell line in an attempt to identify compounds that induce collateral sensitivity or display novelty and effectiveness in their ability to effect cancer cell lines *in vitro*.

The seventeen commercially available compounds were screened using the JANUS liquid handling robot, against the MDA16 and MDA-MB-468 cell lines at a concentration of 10μM. Drug treated cells were incubated in the presence of each drug for 48 hours. After this time-point, the alamar blue assay was performed to measure drug-induced changes in cellular reductase activity. A compound that met the outlined aims i.e caused collateral sensitivity or that displayed novelty at decreasing cellular reductase, must decrease cellular reductase activity by 50% in order to be selected for further characterisation. The antineoplastic dacarbazine was the least effective at decreasing cellular reductase activity in the secondary screen compared to its initial identification from the JHCCL (Figure 3.6A). Despite decreasing cellular reductase activity mitoxantrone, colchicine and mebendazole have been reported to possess cytotoxic activities *in vitro* and as such were not selected for further characterisation. The antineoplastics cytarabine, gemcitabine and cladribine decreased cellular reductase activity of the MDR-TNBC cell line MDA16 more effectively than the MDA-MB-468 cell line (Figure 3.6A). All three compounds decreased cellular reductase activity of the in the MDA16 cell line by greater than 50%. The non-neoplastic compounds illustrated varying degrees of effectiveness at decreasing cellular reductase activity. The anti-bone resorptive agent pamidronate was the least effective non-neoplastic agent at decreasing cellular reductase activity (Figure 3.6B). The antimalarial quinacrine (QC) and the antibiotic anisomycin (ANS), were found to be highly effective in their ability to decrease cellular reductase activity against both the MDA-MB-468 and MDA16 TNBC cell lines. The antifungal ciclopirox olamine (CPX), the antibiotic nitroxoline and the immunosuppressant mycophenolic acid (MPA), effectively decreased cellular reductase activity against both the MDA-MB-468 and MDA16 TNBC cell lines. The activity of these compounds against the TNBC cell lines was at the time of screening, novel (Figure 3.6B).

The ability of compounds tested in the secondary screen to caused collateral sensitivity i.e. the MDA16 cell line is more sensitive to a compound compared to the MDA-MB-468 cell line, was graphed by expressing the decrease in cellular reductase activity in the MDA-MB-468/MDA16 cell lines as a ratio. The antimetabolites cytarabine and gemcitabine are well established to cause collateral sensitivity. Cytarabine was the least effective of the three antimetabolites and therefore it was determined that any compound causing collateral sensitivity with equal or greater effectiveness as cytarabine would be selected for further characterisation. This effectiveness equated to a ratio greater than 1.5. Compounds with a ratio of less than 1 illustrated that the MDA-MB-468 cell line is more sensitive than the MDA16 cell line. The most effective compounds were both cladribine

and gemcitabine (Figure 3.6C). None of the non-antineoplastic agents were found to cause collateral sensitivity (Figure 3.6D). The ability of cladribine to cause collateral sensitivity is a novel finding and thus was selected for further characterisation.

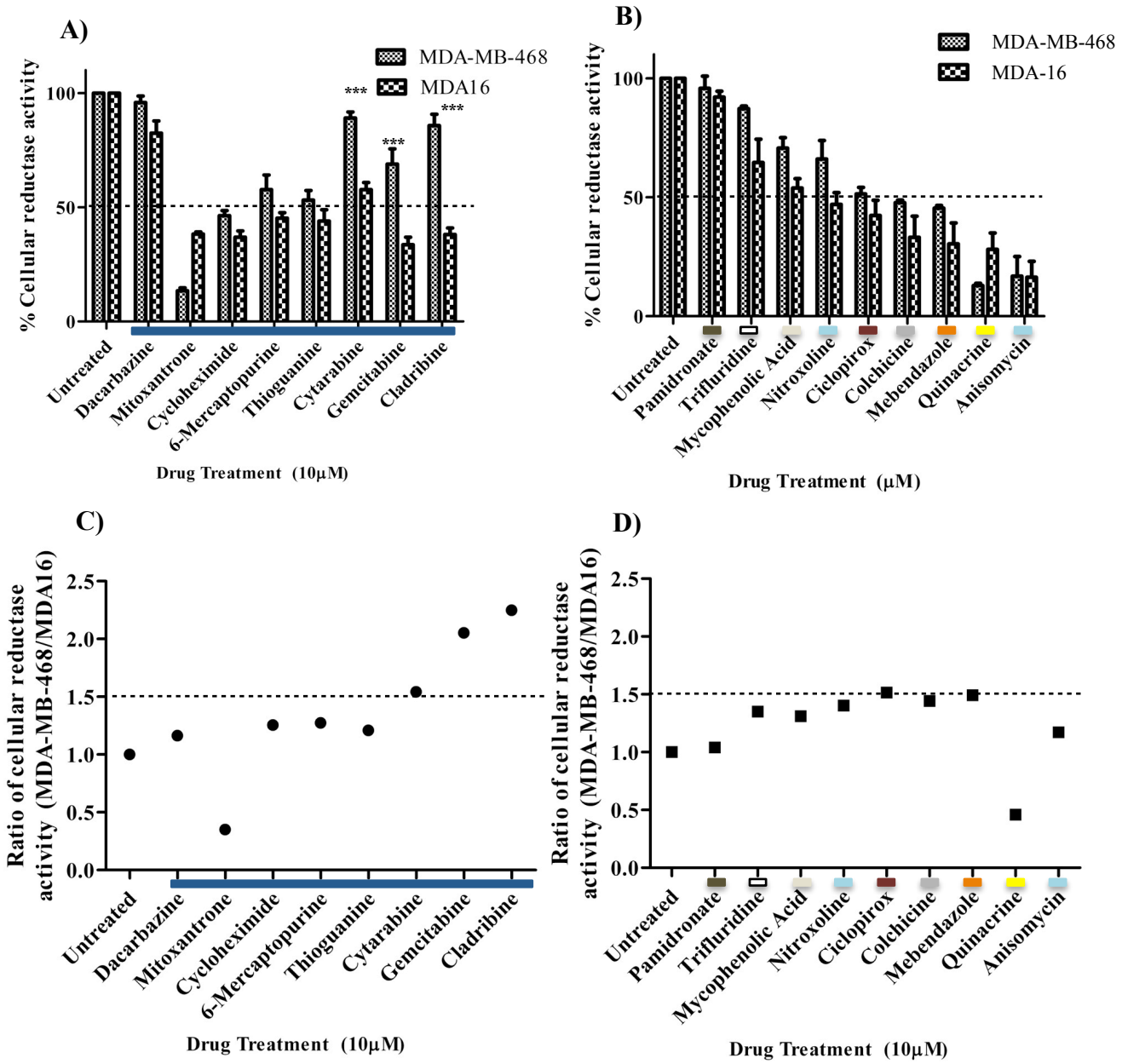


Figure 3.6 Secondary screening of hit compounds independent of the JHCCL - Secondary screening was performed at a concentration of 10μM on the TNBC cell lines MDA-MB-468 and the MDR derivative MDA16. Broken line represents cut off point for compounds that cause collateral sensitivity and compounds that decrease cellular reductase activity in both MDA-MB-468 and MDA16 cell lines. **A)** The antineoplastic compounds cladribine, gemcitabine and cytarabine are selectively more effective at decreasing cellular reductase activity in the MDA16 cell line, compared to the MDA-MB-468 cell line. **B)** No non-antineoplastic compounds caused collateral sensitivity. Compounds that decrease cellular reductase activity of both the MDA-MB-468 cell line by greater than 50% would be considered for further characterisation. **C)** antineoplastic and **D)** non-antineoplastic ability to cause collateral sensitivity is shown by expressing the decrease in cellular reductase activity as a ratio of MDA-MB-468:MDA16. Values greater than one illustrate that the MDA16 cell line was more sensitive. Values less than one illustrate that the MDA-MB-468 cell line was more sensitive. The antimetabolites cytarabine, gemcitabine and cladribine were identified to cause collateral sensitivity. Broken line represent cut-off point for a compound that induced collateral sensitivity and was selected by the activity of cytarabine, a compound known to cause collateral sensitivity. (Data shown n=3, ± SEM. Statistically significant differences between MDA-MB-468 and MDA16 cell lines in response to drug treatment determined by two way ANOVA, followed by Bonferroni post hoc test, ***P<0.001.

ANS, CPX, QC, MPA and nitroxoline, did not cause collateral sensitivity, but showed effectiveness and/or novelty in their ability to decrease cellular reductase activity were selected for further characterisation. At the time of screening, nitroxoline had not been reported to possess cytotoxic activities against cancer cell lines *in vitro* or *in vivo*. Due to its potential novelty, nitroxoline was selected as a compound of interest for further characterisation. In order to determine its effectiveness, a 48 hour nitroxoline concentration response was performed using the MDA-MB-468 and the MDA16 TNBC cell lines. Nitroxoline was found to induce a statistically significant decrease in cellular reductase activity in both parental MDA-MB-468 and the MDR MDA16 TNBC cell lines (Figure 3.7A). There was no statistically significant difference between the response of the two cell lines to nitroxoline. Despite this potentially interesting and novel result, nitroxoline is reported to possess a poor pharmacokinetic profile *in vivo* due to its short plasma half life. Nitroxoline accumulates in the bladder and is rapidly excreted through the urine (Mrhar et al., 1979). These data suggest that, if nitroxoline successfully made it past the preclinical stage and into clinical trials, it is likely that its poor pharmacokinetic profile would prevent the achievement of pharmacologically relevant concentrations in the plasma. Due to this, in addition to the identification of more effective and interesting compounds, nitroxoline was not selected for further characterisation *in vitro.*

Mycophenolic acid (MPA) has previously been reported to possess cytotoxic activities against a number of different cancer cell lines *in vitro* and in preclinical animal models. However, MPA has not previously been reported to possess cytotoxic activities against breast cancer cell lines *in vitro*. Thus, MPA was selected for further characterisation and represented a compound on the threshold of the cut-off criteria outlined in the secondary screen. A 48 hour MPA concentration response using the MDA-MB-468 and MDA16 cell lines was performed. MPA was found to be relatively ineffective at decreasing cellular reductase activity compared to some of the other hit compounds identified, with a similar effect observed in both cell lines. At a concentration of 1μM there was an approximate 50% decreased in cellular reductase activity, at concentrations greater than 1μM there was a plateau in the decrease of cellular reductase activity induced by MPA, even at concentrations of 100μM (Figure 3.7B). Due to the ineffectiveness of MPA to decrease cellular reductase activity and identification of more effective compounds from the secondary screen, MPA was not selected for further characterisation.

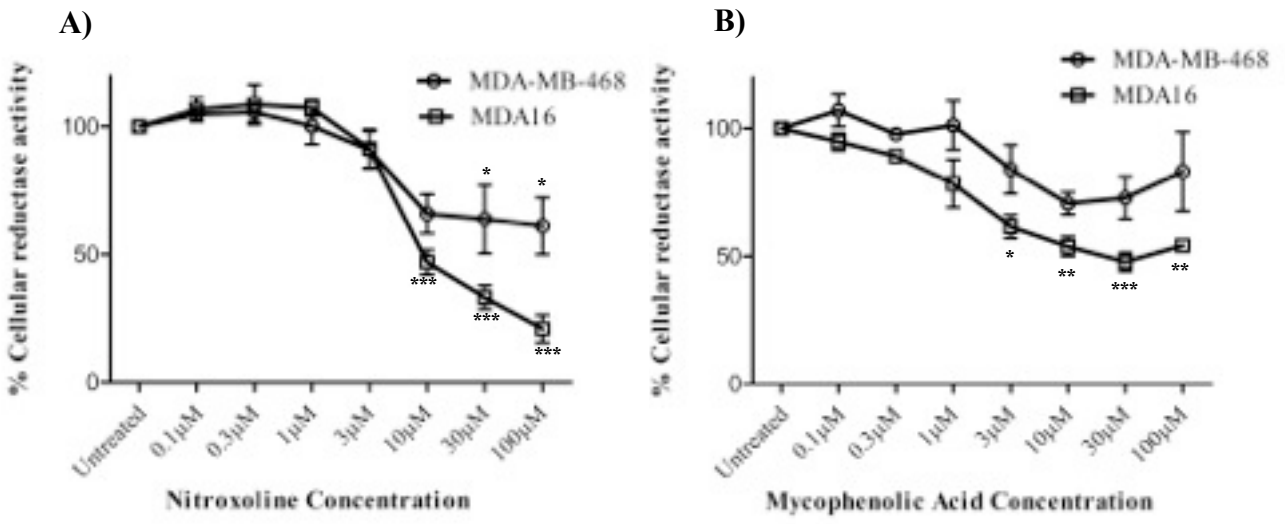


Figure 3.7 Concentration response using the antibiotic nitroxoline and the immunosuppressant mycophenolic acid against the TNBC cell lines MDA-MB-468 and MDA16 - The MDA-MB-468 and MDA16 cell lines were treated with a concentration response using either nitroxoline or mycophenolic acid and cells were incubated with the drug for 48 hours, after this time the alamar blue assay was performed. **A)** Nitroxoline decreased cellular reductase activity in both the MDA-MB-468 and MDA16 cell lines with an EC_{50} of ~10μM. **B)** Mycophenolic acid decreased cellular reductase activity in both the MDA-MB-468 and MDA16 cell lines but with limited effectiveness. (Data shown n=3, ± SEM, comparing each drug concentration to untreated control, two way ANOVA followed by Bonferrnoi post hoc test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

Five out of the nine antineoplastic compounds identified from the primary screen were antimetabolites, one of which is cladribine. Cladribine was identified as one of the most effective antineoplastic compound in the secondary screen and interestingly cladribine was associated with collateral sensitivity. In the secondary screen 10μM cladribine induced a statistically significant decrease in cellular reductase activity in the MDR-MDA16 cell line, but failed to significantly decrease cellular reductase activity in the parental "sensitive" MDA-MB-468 cell line (Figure 3.8A). Due to the selectivity of cladribine-induced decrease in cellular reductase activity against the MDR1 over-expressing MDA16 cell line, a cladribine concentration response was performed using MCF-7 and a derivative MCF-7 cell line that has over-expressed MDR1. Both cell lines were incubated with cladribine for 48 hours and after this time the alamar blue assay was performed. Cladribine was shown to induce a statistically significant decrease in cellular reductase activity in the MCF-7 cell line over-expressing MDR1, with a maximal decrease in cellular reductase activity of 50% at a concentration of 1μM. The MCF-7 cell line did not show a decrease in cellular reductase activity, even at concentrations of 100μM (Figure 3.8B).

A panel of TNBC cell lines that are not reported to over-express MDR1, were screened with cladribine to test the selectivity of cladribine-mediated decrease in cellular reductase activity observed in the MDA16 and MDR1 over-expressing MCF-7 cell lines. A 48 hour concentration response of cladribine was performed on three TNBC cell lines; BT20, HCC1937 and Hs578t. Cellular reductase activity of the three TNBC cell lines tested was not decreased in response to cladribine treatment even at concentrations of 100µM (Figure 3.8C). These results suggest collateral sensitivity to cladribine is caused by the over-expression of MDR1.

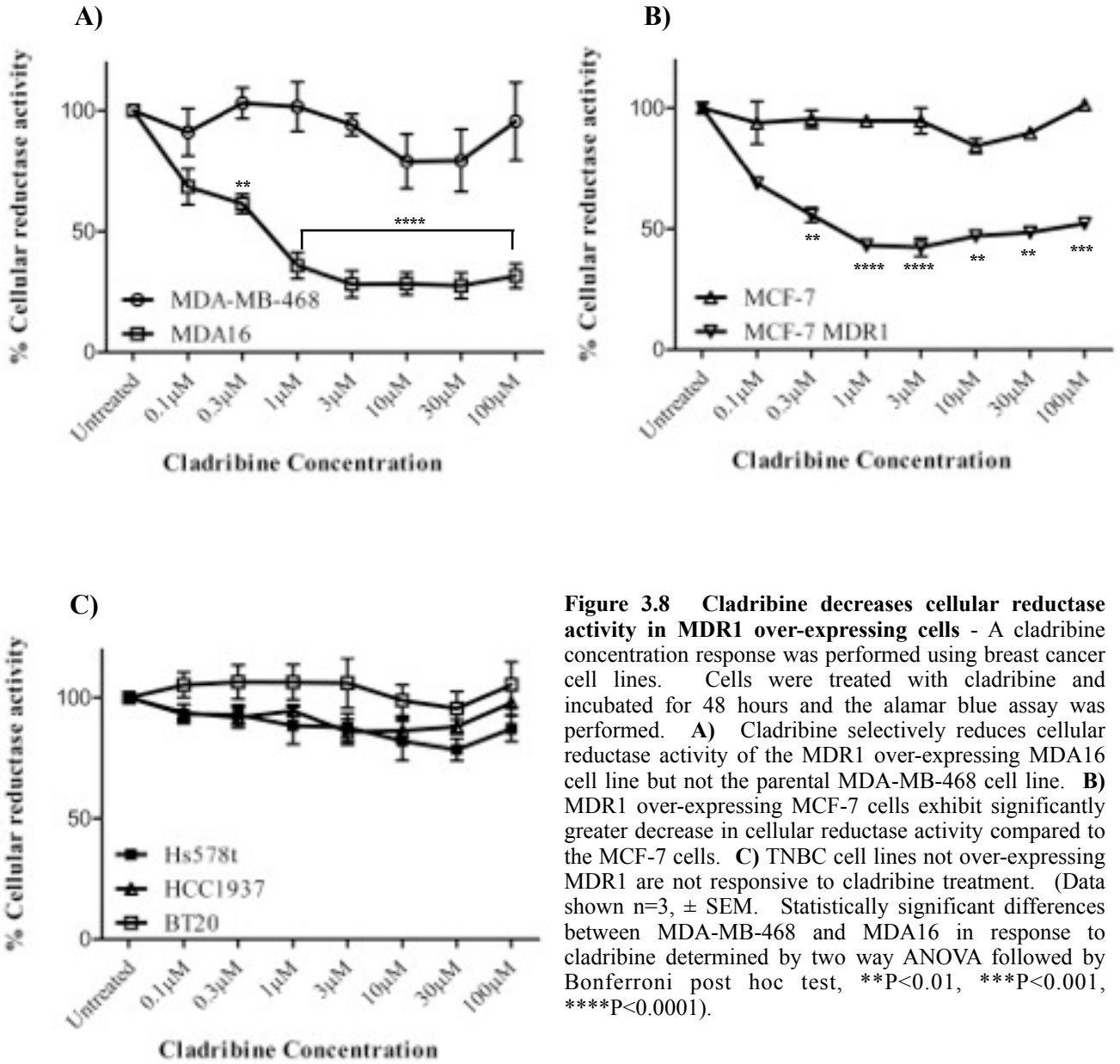


Figure 3.8 Cladribine decreases cellular reductase activity in MDR1 over-expressing cells - A cladribine concentration response was performed using breast cancer cell lines. Cells were treated with cladribine and incubated for 48 hours and the alamar blue assay was performed. **A)** Cladribine selectively reduces cellular reductase activity of the MDR1 over-expressing MDA16 cell line but not the parental MDA-MB-468 cell line. **B)** MDR1 over-expressing MCF-7 cells exhibit significantly greater decrease in cellular reductase activity compared to the MCF-7 cells. **C)** TNBC cell lines not over-expressing MDR1 are not responsive to cladribine treatment. (Data shown n=3, ± SEM. Statistically significant differences between MDA-MB-468 and MDA16 in response to cladribine determined by two way ANOVA followed by Bonferroni post hoc test, **P<0.01, ***P<0.001, ****P<0.0001).

QC, an antimalarial, was the second most effective drug identified from the secondary screen, that decreased cellular reductase activity. A 48 hour QC concentration response was performed on the MDA-MB-468 and MDA16 TNBC cell lines. QC decreased cellular reductase activity equally between the MDA-MB-468 and MDA16 cell lines, with an EC_{50} of approximately 5µM (Figure 3.9A). The selectivity of QC-mediated decrease in cellular reductase activity was tested by performing a concentration response against the non-cancer breast cell line MCF10a. QC was found to effectively decrease cellular reductase activity in the non-cancer breast cell line MCF10a, with an EC_{50} of 10µM, a similar concentration required for QCs activity against the TNBC cell lines (Figure 3.9B). Due to QCs lack of selectivity between cancer and non-cancer cell lines *in vitro,* in combination with the lack of identifiable collateral sensitivity, QC was not selected for further characterisation.

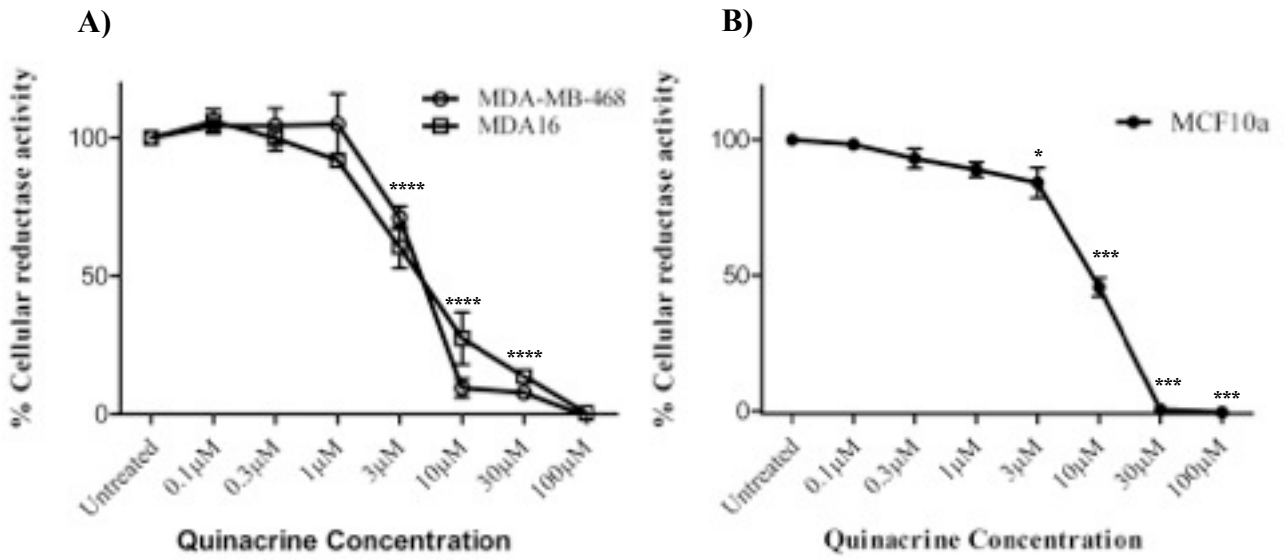


Figure 3.9 The antimalarial QC non-selectively decreases cellular reductase activity of TNBC and non-cancer cell lines - A 48 hour QC concentration response was performed on the **A)** MDA-MB-468 and MDA16 TNBC and **B)** the MCF10a non-cancer breast cell lines. QC non-selectively decreased cellular reductase activity in the MDA-MB-468, MDA16 and MCF10a cell lines with an EC_{50} of approximately 10 µM. (Data shown n=3, ± SEM, comparing each concentration to untreated control, two way ANOVA followed by Bonferrnoi post hoc test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

The antibiotic ANS was the most effective compound identified in the secondary screen, where at a concentration of 10µM, ANS decreased cellular reductase activity of both the MDA-MB-468 and MDA16 cell lines to approximately 20% (Figure 3.6B). Performing an ANS concentration response, illustrated that ANS induced a statistically significant decrease in cellular reductase activity, with an EC_{50} of approximately 300nM in the MDR-MDA16 and parental MDA-MB-468 TNBC cell lines (Figure 3.10A). The effectiveness of ANS to decrease cellular reductase activity in the MDA-MB-468 cell line is comparable to the effectiveness of doxorubicin. More significantly however, is the ability of ANS to effectively decrease cellular reductase activity of the MDR-TNBC cell line MDA16. Due to ANS's effectiveness, it was selected for further characterisation in an attempt to determine its spectrum of activity, mechanism of action and the mode of cell death induced by ANS. The antifungal CPX was identified from the secondary screen, as a compound that effectively decreased cellular reductase activity to less than 50% in both the MDA-MB-468 and MDA16 cell lines at a concentration of 10µM (Figure 3.6B). Performing a CPX concentration response against the MDA-MB-468 and the MDA16 cell lines, illustrated that CPX was an effective compound at decreasing cellular reductase activity with an EC_{50} of approximately 3µM in the MDA16 cell line and approximately 20µM in the MDA-MB-468 cell line (Figure 3.10B). Due to the novelty and effectiveness of CPX at decreasing cellular reductase activity against the MDA-MB-468 and MDA16 cell lines, it was selected for further characterisation

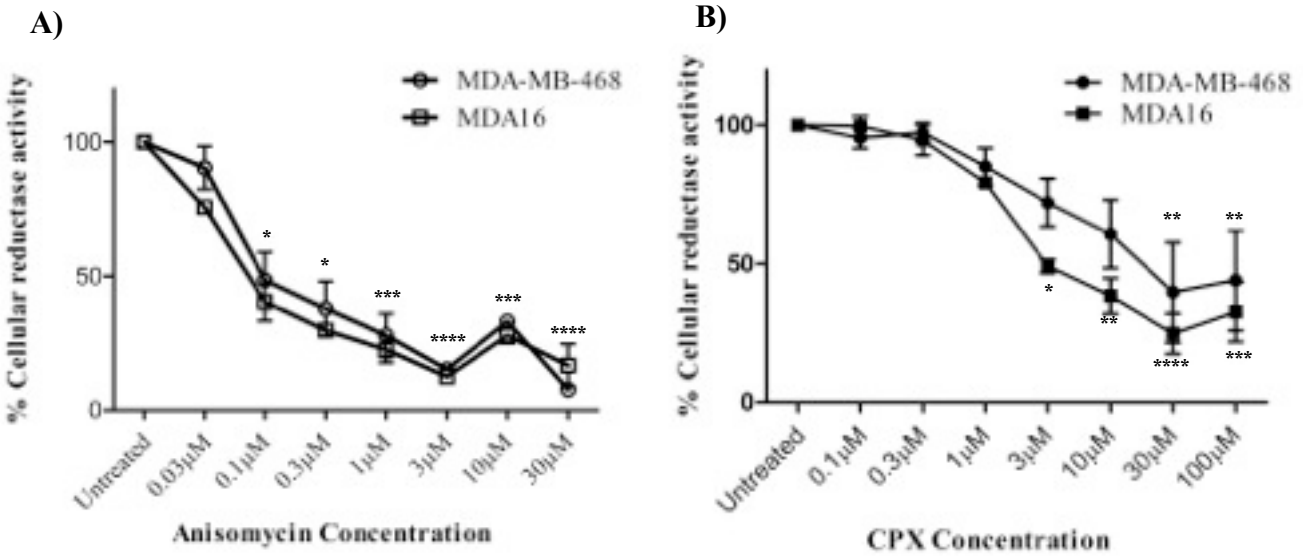


Figure 3.10 The antibiotic ANS and the antifungal CPX effectively decrease cellular reductase activity in the TNBC cell lines MDA16 and MDA-MB-468 - An ANS and CPX concentration response was performed on the MDA-MB-468 and MDA16 cell lines. Cell were incubated in the presence of each drug for 48 hours, after this time the alamar blue assay was performed. **A)** ANS is highly effective at reducing the cellular reductase of the two TNBC cell lines tested. ANS possess an EC_{50} of approximately 100nM. **B)** CPX significantly reduces cellular reductase activity in both MDA-MB-468 and the MDA16 TNBC cell lines. (Data shown n=3, ±SEM, comparing each concentration to untreated control, two way ANOVA followed by Bonferroni post hoc test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

3.3 Discussion

3.3.1 *Identification of hit compounds from the primary screen of the JHCCL*

Before the JHCCL screen was performed, the reported MDR phenotype of the MDA16 cell line was confirmed. In addition, the alamar blue assay was optimised for HTS to ensure that the most robust assay format was employed, increasing the likelihood of identifying a true hit and not a false positive (Zhang et al., 1999). A Z-value of 0.95 was achieved, illustrating that there was tight control of the variables in the alamar blue assay.

The primary screen identified thirty unique compounds, that decreased cellular reductase activity by greater than three standard deviations from the mean, a prerequisite outlined before carrying out the JHCCL screen. Compounds classified as dyes, diagnostics and antiseptics were omitted from the list of compounds to acquire for secondary screening (Table 2). Clinically, most of these compounds are administered topically and thus, the availability of preclinical and clinical pharmacological and toxicological data relating to systemic administration would not be available. However, performing a retrospective literature search on the class of dyes and diagnostics, identified that two dyes; methylene blue and toluidine blue, have been tested *in vitro* against prostate cancer cell lines and both compounds exhibited selectively towards cancer cell lines. Methylene blue and toluidine blue belong to the same class of drug; phenothiazines. Phenothiazines are known to interfere with redox cycles and it is reported that the sensitivity of some cancer cell lines to these compounds is due to increased NADPH:quinone oxidoreductase enzymatic activity observed in the sensitive cancer cells. Treating cancer cell lines that possess increased NADPH:quinone oxidoreductase enzymatic activity, with either methylene blue or toluidine blue, induced oxidative stress and subsequently apoptosis (Wondrak, 2007). In addition to effecting the NADPH:quinone oxidoreductase system, methylene blue is reported to inhibit both monoamine oxidase A, a cellular reductase enzyme that degrades the neurotransmitters serotonin and noradrenaline, (Petzer et al., 2012) and guanylate cyclase, an enzyme responsible for generation of cyclic guanosine monophosphate from cyclic guanosine triphosphate (Mayer et al., 1993). Increased expression of mutated guanylate cyclase is associated with increased proliferation of prostate cancer cell lines *in vitro* (Mujoo et al., 2010) and inhibition of this activity pharmacologically or using siRNA reduced cell number (Cai et al., 2007).

Elevated expression of monoamine oxidase A, is associated with high grade prostate cancer in human tumours (Peehl et al., 2008). Pharmacological inhibition of monoamine oxidase A, decreases prostate cancer cell growth *in vitro* and reduced prostate cancer tumour growth in preclinical xenograft models (Flamand et al., 2010). Thus, identification that methylene blue effectively decreases cellular reductase activity in the MDR-TNBC cell line MDA16 is a novel finding. Characterising the spectrum of methylene blue effects against a panel of cancer cell lines, as well as testing the functionality of the molecular targets reported in the literature, will strengthen the current knowledge of methylene blue as a cytotoxic agent *in vitro* and may result in the translation of this compound for the treatment of patients with TNBC.

After excluding dyes, diagnostics and antiseptics twenty four hit compounds remained. A further seven compounds were excluded from validation in the secondary screen, which included the cardiac glycosides strophanthin K, proscillardin and acetyldigitoxin, the two emetics emetine and ipecac syrup, the antineoplastic diaziquone and the dermatologic podophyllin resin.

Podophyllin resin is a topical cream used for the removal of genital warts (Scheinfeld and Lehman, 2006). It contains a mixture of active compounds that contribute to its clinical activity. Podophyllin resin has not previously been reported to possess cytotoxicity activities against cancer cell lines *in vitro* or anticancer activities *in vivo*. Due to podophyllin resins formulation as a topical cocktail of active compounds, identification and purification of the specific components that contribute to the effective decrease in cellular reductase activity observed in the primary screen on the MDA16 cell line would be difficult. Indeed, it may be that a number of active compounds within the cocktail are required to interact, affecting multiple cellular pathways in the cancer cell that together, contribute to decreasing cellular reductase activity. Due to this, podophyllin resin was omitted from the list of compounds to validate in the secondary screen.

Cardiac glycosides are known to inhibit the Na^+/K^+ ATPase pump, resulting in increased intracellular levels of calcium, which is required for their pharmacological activity. Despite being effective in the treatment of congestive heart failure, cardiac glycosides have an extremely narrow therapeutic window and are associated with significant dose limiting side effects including nausea, fatigue, headache, blurred vision and dyspnea (Newman et al., 2008).

There is extensive data in the literature reporting that cardiac glycosides such as strophanthin K, and ouabain possess cytotoxic activities *in vitro* against breast, lung, leukemic and prostate cancer cell lines (Newman et al., 2008). Despite possessing potent cytotoxic activities against cancer cell lines *in vitro,* testing of the cardiac glycoside oleander in a phase I clinical trial against solid tumours reported no antitumour effect (Mekhail et al., 2006). This lack of antitumour activity, is now thought to be associated with the low dose used to avoid toxicity issues and the intramuscular route of administration. A newer formulation of oleander, known as PB-05204, has been specifically formulated for oral administration and is in a phase I clinical trial testing its potential anticancer activity against solid tumours and is due to conclude in October 2012 (Hong, 2007).

Strophanthin K was represented twice in the JHCCL drug plate, one decreased cellular reductase activity in the MDA16 TNBC cell line by over 80%, while the second decreased cellular reductase activity by less than 40%. The difference between the two compounds in the library is uncertain, despite this strophanthin K was not available commercially. Ouabain, a related analogue of strophanthin K, was obtained instead. Performing a ouabain concentration response using the MDA-MB-468 and MDA16 cell lines, resulted in a statistically significant decrease in cellular reductase activity in both cell lines. This data is not shown as ouabain was not identified by screening the JHCCL. Due to the known toxicity issues associated with cardiac glycosides, the lack of clinical efficacy and lack of novelty relating to cardiac glycosides possessing cytotoxic effects against cancer cell lines, no further characterisation of this drug class was performed.

Both emetine and ipecac syrup are primarily used in a clinical setting to induce emesis. Ipecac syrup, similar to podophyllin resin, is a complex mixture of active compounds. Thus, identification and purification of the key compound(s) in the mixture, that contribute to the decrease in cellular reductase activity observed in the primary screen would be extremely difficult. Due to this, ipecac syrup was not selected for further characterisation. Aside from its emetic indication, emetine has also been used clinically as an antiprotozoal, where its mechanism of action relies on inhibition of protein synthesis (Grollman, 1966, Zierdt et al., 1983). The anticancer activity of emetine has been reported for almost a century. Results from a study conducted in 1918 report that, from one hundred patients with various forms of solid tumours treated with emetine, thirty-five patients that had either breast or cervical cancer responded (Cushny, 1918). Despite exhibiting effective anticancer activities, eight patients died from “exhaustion” and three from cardiac arrest, thus illustrating that emetine has a potentially narrow therapeutic window.

A more recent study using emetine against solid tumours, report that emetine is associated with significant side effects and has limited antitumour activity (Mastrangelo et al., 1973). These data suggest that in order for the anticancer activities of emetine to be exploited, identification of its target in tumours is essential for stratifying patients that are likely to respond to emetine based therapy. Due to the lack of effectiveness in the primary screen, in addition to the lack of novelty, emetine was not selected for further characterisation.

Finally, the antineoplastic diaziquone is an alkylating agent that induces DNA cross linking and subsequent DNA strand breaks. Due to the ability of diaziquone to cross the blood brain barrier, it was used extensively against primary brain tumours (Bender et al., 1983), but also shows anticancer activity against relapsed non-lymphocytic leukemia (Lee et al., 1986). As diaziquone is a known antineoplastic agent and exhibited a limited ability to decrease cellular reductase activity against the MDA16 cell line, it was not selected for further characterisation.

Thus, all of the above compounds identified in the primary screen, with the exception of podophyllin resin and ipecac syrup, have been previously reported to either possess cytotoxic activities against cancer cell lines *in vitro* and in the cases of emetine, diaziquone and some of the cardiac glycosides, possess at least some antitumour activity in patients. While it would have been ideal to validate the findings exhibited by these compounds induced in the primary screen using compounds sourced independent from the JHCCL, it was decided to select some of the more novel and interesting compounds.

3.3.2 Secondary screening hit compounds and validation

The secondary screen was performed by screening the seventeen compounds obtained independently of the JHCCL, at a concentration of 10μM, on the parental MDA-MB-468 and the MDR-TNBC MDA16 cell lines. The hit compound selection process is outlined in Figure 3.5. One aim of the screen, was to identify a compound(s) that exhibited collateral sensitivity i.e. the MDA16 cell line would be more sensitive to drug-induced reduction in cellular reductase activity. If a collaterally sensitive causing compound was identified, the activity exhibited must be both novel and effective. Compounds exhibiting collateral sensitivity, whose activity was neither novel nor effective would not be selected for further characterisation. In addition to identifying compounds that caused collateral sensitivity, compounds that did not induce collateral sensitivity, but exhibited either novel and/or effective activities against both the MDA-MB-468 and MDA16 cell lines, would be considered for selection for further characterisation. However, this compound must decrease cellular reductase activity by greater that 50% in both cell lines. Pamidronate and dacarbizine were identified as the least effective compounds at reducing cellular reductase activity in both cell lines, suggesting that these two compounds were false positives from the primary screen. It is worth noting that while attempting to obtain the thirty hit compounds identified from the primary screen, it was found that two hit compounds were mislabeled in the JHCCL drug plate. The well containing the dye brilliant blue, on the JHCCL drug plate was also labelled brivudine, an antiviral drug and the well denoting the dye tolonium blue, was also labelled tolonidine, an antihypertesive. Despite this, the remaining compounds identified by their ability to decrease cellular reductase activity in the primary screen, when obtained and screened independent from the JHCCL, retained comparable or increased effectiveness at decreasing cellular reductase activity in either the MDA-MB-468 or MDA16 cell lines.

3.3.2.1 *Collateral sensitivity and antimetabolites*

Performing an unbiased screen of the JHCCL against the MDR-TNBC MDA16 cell line identified six antimetabolite compounds. Cladribine, gemcitabine, cytarabine, thioguanine and 6'-mercaptopurine, are well established antineoplastic agents used to treat breast and ovarian cancers and various forms of leukemias. Trifluridine is an antiviral, used to treat herpes and is an analogue of the anticancer agent 5-FU. Trifluridine was identified as a compound on the cut-off point for consideration as a collateral sensitivity causing agent.

However, despite the MDA16 cell line being more sensitive, trifluridine decreased cellular reductase activity by less than 20% and was thus, not effective. Due to this trifluridine was omitted from further characterisation. Thioguanine and 6'-mercaptopurine failed to induce collateral sensitivity and were also ineffective at decreasing cellular reductase activity. Collateral sensitivity was observed with three of the antimetabolites cladribine, cytarabine and gemcitabine in the MDA16 cell line. Gemcitabine, cytarabine and cladribine are prodrugs that require phosphorylation and activation by the same rate limiting enzyme, deoxycytidine kinase (dCK). 2'2'-difluorodeoxycytidine triphosphate and 1-β-D-arabinofuranosylcytosine triphosphate are the active metabolites of gemcitabine and cytarabine respectively. These active metabolites incorporate into DNA and RNA inhibiting the synthesis of both and subsequently induce apoptosis. Cytarabine and gemcitabine have previously been reported to cause collateral sensitivity in MDR1 and MRP1 over-expressing cancer cell lines. Selective culturing of small cell lung carcinoma (SCLC) cell lines in a panel of cytotoxic agents, generated daughter cell lines whereby one cell line was sensitive and the other cell line, due to culturing in increasing concentrations of the particular cytotoxic agent, was resistant. Cytarabine caused collateral sensitivity to SCLC cell lines selectively resistant to daunorubicin, etoposide, teniposide, cisplatin and carmustin. Interestingly, gemcitabine caused collateral sensitivity only in SCLC cells resistant to daunorubicin and carmustin ***(Jensen et al., 1997)***. The resistant cell lines were shown to have elevated MDR1 and MRP1 protein expression. Collateral sensitivity to gemcitabine has also been illustrated in non-SCLC, ovarian and melanoma cancer cell lines that selectively over-express either MDR1 or MRP1 by selective culturing in doxorubicin or transfecting MDR1 into cells (Bergman et al., 2001).

An association between over-expression of both MDR1 or MRP1 and elevated expression of dCK, with a concurrent reduction in deoxycytidine deaminase expression, the enzyme responsible for the metabolism and subsequent inactivation of antimetabolites such as cladribine, gemcitabine and cytarabine in cancer cell lines has been reported (Bergman et al., 2001). The increased expression of dCK and concurrent decrease of deoxycytidine deaminase is believed to be the mechanism responsible for collateral sensitivity observed in MDR1 over-expressing cancer cells to antimetabolite prodrugs that require activation by dCK. The elevated levels of dCk mean cancer cells over-expressing MDR1 or MRP1 activate the prodrugs to their pharmacologically active metabolite, concurrently the cell detoxification system is perturbed due to the associated down-regulation of deoxycytidine deaminase levels.

These changes result in elevation and the persistence of activated drug inside the cell, allowing for cytotoxic levels of the drug to accumulate resulting in cell death. To strengthen this argument, pharmacological inhibition of MDR1 activity using verapamil, reverses collateral sensitivity caused by cytarabine and gemcitabine in MDR1 over-expressing cancer cells *in vitro* (Bergman et al., 2003).

The novel finding from our screen, was the identification of collateral sensitivity observed in the MDR1 over-expressing TNBC cell line MDA16 and the MDR1 over-expressing MCF-7 cell line, in response to cladribine treatment (Figure 3.8A and B). In addition, collateral sensitivity to cladribine, gemcitabine and cytarabine has not previously been reported in breast cancer cell lines. Cladribine has an EC_{50} of approximately 1μM in both the MDA16 and MDR1 over-expressing MCF-7 cell lines, with no significant decrease in cellular reductase activity detected in either of the parental cell lines. In addition, the non-MDR1 over-expressing TNBC cell line did not respond to cladribine treatment. This data suggests that cladribine, gemcitabine and cytarabine may be potentially useful compounds to treat breast cancers that over-express MDR1. Indeed, development of imaging techniques such as the fluorescent MDR1 substrate sestamibi, allow for detection of cancers over-expressing MDR1 in patients (Agrawal et al., 2003, Ciarmiello, 1998) and can be employed to measure the effectiveness of compounds that exhibit cytotoxic activities against these cancers *in vitro*.

Despite this novel finding, extensive literature is available describing collateral sensitivity caused by cytarabine and gemcitabine in other MDR1 and MRP1 over-expressing cancer cell lines and is thus, already associated with this drug class. Due to this, further characterisation of these compounds was not performed. If further characterisation of the collateral sensitivity caused by cladribine is performed in the future, it may be interesting to measure the relative expression levels of MDR1 alongside the expression and activity of both dCk and deoxycytidine deaminase between the MDA16 and MDR1 over-expressing MCF-7 breast cancer cell lines.

3.3.2.2 *Spectrum of nitroxoline, mycophenolic acid and quinacrine activities against a panel of TNBC cell lines*

The antibiotic nitroxoline, the immunosuppressant MPA and the antimalarial QC, were validated in the secondary screen to significantly decrease cellular reductase activity. At the time of screening, there was conflicting data relating to MPA possessing cytotoxic activities against cancer cell lines *in vitro* and in preclinical models. Nitroxoline had not previously been linked to possess cytotoxic activities against cancer cell lines *in vitro.* Conversely, the cytotoxic activities of QC against a range of cancer cell lines has been extensively reported in the literature for almost two decades, however the spectrum and mechanism of QCs cytotoxic activities against cancer cell lines *in vitro* is poorly characterised.

The current clinical application of nitroxoline is for the treatment of bacterial and fungal infections of the urinary tract. Nitroxoline has been reported to possess a very short plasma half life of just under three hours. Following oral administration of a Nibiol tablet containing 100mg nitroxoline, plasma concentrations peaked at a concentration of 5.5µg/ml after two hours, this equates to approximately 30µM (Ghoneim et al., 2011). However, nitroxoline is rapidly cleared from the plasma and accumulates in the bladder (Bergogne-Berezin et al., 1987, Ghoneim et al., 2011, Mrhar et al., 1979). The EC_{50} of nitroxoline is approximately 10µM in both the MDA-MB-468 and MDR MDA16 TNBC cell lines. Despite nitroxoline effectively decreasing cellular reductase activity in both of the TNBC cell lines tested, it was not selected for further characterisation due to its known poor pharmacokinetic profile *in vivo*. Interestingly, following its identification in the primary screen, nitroxoline was reportedly identified from two independent unbiased screens performed by the same group (Shim et al., 2010). A library of 175,000 compounds was screened, in an attempt to find inhibitors of the known angiogenesis factor MetAP2 and identified nitroxoline as the most effective compound. In addition, screening the JHCCL against the human umbilical vein endothelial cord (HUVEC) cell line, identified nitroxoline as an effective inhibitor of cell proliferation determined by the reduction of thymidine incorporation into DNA. Further characterisation of nitroxoline uncovered antiangiogenic and antiproliferative activities *in vivo,* as evidenced by reduced tumour growth in preclinical xenograft models of breast and bladder cancers (Shim et al., 2010).

Furthermore, nitroxoline was identified in a screen of 2816 clinically approved compounds from the Health Chemical Genomic Centre's pharmaceutical collection, as the most effective compound to reduce cellular reductase activity in the thyroid cancer cell line TPC-1 (Zhang et al., 2011). Since our initial identification of nitroxoline in the primary screen, it has been reported to possess cytotoxic activities against bladder, breast and thyroid cancer cell lines *in vitro* and against bladder and breast cancer xenograft models *in vivo.*

MPA, is a known inhibitor of inosine monophosphate dehydrogenase, the rate limiting enzyme in the *de novo* synthesis of guanine nucleotides. MPA is an immunosuppressant, used clinically for prophylaxis of allograft rejection following renal, cardiac and liver transplants (Villarroel et al., 2009). MPA was validated in the secondary screen and represents a compound that was on the cut-off threshold i.e. ≧50% decrease in cellular reductase activity. The results of the alamar blue assay concentration response, suggest that MPA may induce a cytostatic effect in the MDA-MB-468 and MDA16 cell lines, due to the plateau in cellular reductase activity observed at drug concentrations greater than 3μM. This correlates with the proposed mechanism of MPA activity in the prostate cancer cell line DU145, where MPA was shown to induce cell cycle arrest (Floryk and Huberman, 2006). MPA has been reported to inhibit angiogenesis *in vitro,* using the standard angiogenic assays of endothelial cell migration, with subsequent tube formation and aortic ring formation (Domhan et al., 2008, Koehl et al., 2007). Induction of apoptosis in response to MPA treatment has been reported in multiple myeloma cancer cell lines and against primary multiple myeloma cells *in vitro* (Takebe et al., 2006). Additionally, MPA inhibited the growth of murine colon adenocarcinoma and melanoma cancer cell lines and against human gastric adenocarcinoma, prostate and glioblastoma cancer cell lines *in vitro,* but the mode of cell death was not reported (Koehl et al., 2007, Villarroel et al., 2009). Despite exhibiting cytotoxic and growth inhibitory effects *in vitro* against cancer cell lines, there are some inconsistencies related to the effects of MPA in preclinical xenograft models of cancer. Using athymic BALB/c nude mice as a preclinical xenograft model of murine melanoma and murine and human adenocarcinomas, MPA was found to induce no significant reduction in tumour cell growth compared to control animals (Koehl et al., 2007). This is in contrast to the significant decrease in cell number induced by MPA *in vitro* against these cell lines (Koehl et al., 2007). The inconsistency between the *in vitro* and preclinical results have been attributed to the poor bioavailability of MPA, as it is known to be rapidly glucuronidated and inactivated by UDP-glucuronosyl transferase (Franklin et al., 1996).

Conversely, MPA was found to be ineffective against the glioblastoma cancer cell line U87 *in vitro* but interestingly, in a preclinical model of glioblastoma in BALB/c nude mice using the U87 cell line, MPA induced a significant reduction in tumour growth rate (Domhan et al., 2008). The difference observed between the *in vitro* and *in vivo* data has been linked to the ability of MPA to alter the expression of key matrix remodelling and pro-angiogenesis genes in non-tumour cells present in the tumour microenvironment (Domhan et al., 2008). However, this does not explain the difference between the two studies. A potential explanation relates to the route of administration and dose administered which differed significantly between the studies. In the study using murine melanoma and murine and human adenocarcinomas MPA was administered by intra-peritoneal injection, at a dose of 40-80mg/kg/day. MPA elicited no anti-tumour or growth inhibitory effect in this study. In contrast, the study using the U87 glioblastoma xenograft model, MPA was administered by oral gavage at a dose of 120mg/kg/day. Thus, the difference between the route of administration and the doses administered, may have a significant effect on the final plasma concentration of MPA that can be achieved and only in the U87 glioblastoma xenograft model did MPA reach a level capable of inducing an antitumour effect.

QC's current primary indication is as an antimalarial. The mechanism of action responsible for its antimalarial activity is not well understood. QC was identified from the secondary screen as one of the most effective compounds at reducing cellular reductase activity. Indeed, this data coincides with the published literature relating to QC's cytotoxic activities against cancer cell lines, which has been established for nearly three decades. QC is reportedly cytotoxic against breast, colorectal and MDR leukemic cancer cell lines *in vitro* (Gallant et al., 2011, Preet et al., 2011, Zamora et al., 1988). QC has also been shown to sensitise colorectal cancer cell lines to 5-FU *in vitro*. Additionally, in a preclinical xenograft model of colorectal cancer, QC as a monotherapy decreased tumour growth rate (Gallant et al., 2011). The most important observation from this study however, was the synergistic effect both QC and 5-FU elicited in decreasing colorectal tumour growth rate. These data suggest, that QC may be a useful addition to the current frontline treatment of colorectal cancer, provided dose limiting toxicity does not require the dose of QC to be reduced below the threshold required for its potential anticancer activity. Despite promising results generated using QC against cancer cell lines *in vitro* and in preclinical xenograft models, the molecular target(s) responsible for QC cytotoxic effects is currently not well characterised.

Topoisomerase II has been proposed as a potential target for QC in the breast cancer cell line MCF-7 (Preet et al., 2011), it has also been reported that QC treatment down-regulates NF-kB, increasing the activity of p53 and thereby sensitising renal cell carcinoma cells (Gurova et al., 2005). The lack of a definite marker of sensitivity to QC is likely to become a problem when attempting to stratify patients most likely to respond to QC based intervention. One potentially serious limitation in developing QC as an anticancer agent, is the known organ toxicities that have been reported during its use as an antimalarial. Target organs include the liver and haematological systems as well as reported cases of psychoses (Satoh et al., 2004). Identifying a clinically relevant biomarker of sensitivity would increase the likelihood of QC being repurposed as an anticancer agent. From the *in vitro* and preclinical xenograft data illustrating synergism between QC and 5-FU, it seems that the most likely therapeutic application of QC would be in combination with a current mainstay cytotoxic therapies, providing the desired synergism is not accompanied by a synergism of toxicity.

Due to the poor pharmacokinetic profiles that are reported for both nitroxoline and MPA, in addition to the lack of effect of MPA observed against the MDA-MB-468 and MDA16 TNBC cell lines in this study, these compounds were omitted from further characterisation. QC decreased the cellular reductase activity of both the MDA-MB-468 and MDA16 cell lines with equal effectiveness. However, QC also decreased cellular reductase activity of the non-cancer breast cell line MCF10a at the same concentration responsible for its activity against the cancer cell lines. Due to this lack of selectivity, in combination with its narrow therapeutic window and associated side effects as an antimalarial, QC was not selected for further characterisation.

Ciclopirox olamine is primarily used as an antifungal agent for the treatment of topical mycoses. Prior to its identification in the primary screen, CPX had not previously been reported to possess any cytotoxic effects against cancer cell lines *in vitro* or anticancer properties *in vivo*. CPX was identified from the primary screen as one of the most effective non-antineoplastic compounds, that decreased cellular reductase activity of the MDR-TNBC cell line MDA16. This effect was validated in the secondary screen, as CPX was shown to induce a statistically significant decrease in cellular reductase activity in both the MDA-MB-468 and MDA16 cell lines. The CPX concentration response performed on both cell lines, illustrated that CPX possesses and EC_{50} of 3-6μM. During the process of characterising and validating the effects of CPX, it was reported that CPX effectively inhibited the growth of leukemia cancer cell lines *in vitro* and in preclinical xenograft models (Eberhard et al., 2009).

Despite these findings the ability of CPX to effect breast cancer cell lines *in vitro* was novel. Due to this, CPX was selected for further characterisation in an attempt to determine the spectrum of the activity by testing CPX against a panel of cancer cell lines and determining the mode of cell death induced by CPX in responsive cancer cell lines.

Anisomycin (ANS) is an antibiotic produced by the bacterium *Streptomyces gresolius*. ANS was used in the 1950's for the treatment of intestinal amebesis. However, there are limited clinical reports of ANS from this time on. It appears that the primary application of ANS was as a research tool, for the inhibition of protein synthesis and activation of cellular stress kinases, JNK and p38 (Grollman, 1967, Iordanov et al., 1997, Rolli et al., 1999). ANS was identified from the primary screen as a moderately effective compound at reducing cellular reductase activity. When obtained from a source independent of the JHCCL and tested against the MDA-MB-468 and MDA16 cell lines in the secondary screen, ANS was identified as the most effective compound at decreasing cellular reductase activity. After performing a concentration response, ANS was identified to possess an EC_{50} of approximately 100nM and thus is an extremely effective compound. ANS has previously been reported to sensitise prostate cancer cell lines to Fas mediated apoptosis (Curtin and Cotter, 2002). However, there are no reports of ANS eliciting effects against TNBC cell lines *in vitro*. Thus, due to the novelty of ANS possessing activities against TNBC cell lines *in vitro,* in combination with its effectiveness at decreasing cellular reductase activity, ANS was selected for further characterisation in an attempt to identify the molecular target responsible for its activity and the mode of cell death induced by ANS treatment.

Chapter 4

Investigating and characterising the cytotoxic activity of the antifungal agent Ciclopirox Olamine against cancer cell lines *in vitro*

4.1 Introduction

The antifungal CPX, was identified in the primary screen as one of the most effective non-antineoplastic compounds at decreasing cellular reductase activity of the MDR-TNBC cell line MDA16. A CPX concentration response against the parental TNBC cell line MDA-MB-468 and the MDR daughter cell line MDA16, illustrated that CPX induced similar decreases in cellular reductase activity in both cell lines. Prior to its identification in the primary screen, CPX had not previously been reported to possess cytotoxic activities *in vitro* against cancer cell lines and as such, was selected for further characterisation. Coincidently, following the identification of CPX from the primary screen, it was reported that CPX possesses cytotoxic activities against a panel of leukemic cancer cell lines *in vitro* and this effect was confirmed using preclinical leukemic xenograft models *in vivo* (Eberhard et al., 2009). CPX was reported to induce significant decreases in tumour growth with no reported toxicity issues. In addition, it was reported by a separate group that CPX possessed cytotoxic activities against the TNBC cell line MDA-MB-231 *in vitro* and decreased tumour growth in a preclinical breast cancer xenograft model (Zhou et al., 2010). The mode of cell death induced by CPX in the TNBC cell line was reportedly apoptosis (Zhou et al., 2010). Despite these reports, it was decided to determine the degree of selectivity of CPX's ability to decrease cellular reductase activity by performing a CPX concentration response against a panel of cancer cell lines, to determine if CPX induced apoptosis in the MDA-MB-468 and MDA16 cell lines and attempt to determine CPX's mechanism of action.

4.2 Results

4.2.1 Screening CPX against a panel of cancer cell lines

The spectrum of CPX's ability to decrease cellular reductase activity, was tested by performing a concentration response against a panel of TNBC, non-TNBC, prostate cancer and colorectal cancer cell lines. All cell lines were treated with concentrations of CPX ranging from 0.1µM-100µM and incubated with the drug for 48 hours, after this time the alamar blue assay was performed. These cell lines were selected as they represent the common cancer types in humans. A list of the cancer cell lines and their statuses is outline in appendix 5.

The three prostate cancer cell lines DU145, 22Rv1 and PC3 are all androgen insensitive and thus, do not respond to the mainstay of prostate cancer therapy. Androgen insensitive prostate cancer, similar to TNBC, currently does not possess any targeted therapies. Two of the four colorectal cancer cell lines SW480 and HT29 are p53 mutant and hence express a marker of clinically relevant drug resistance. CPX illustrated a degree of selectivity against a subset of the TNBC cell lines tested. The MDA-MB-231 and MDA16 cell lines were equally sensitive with a 3μM EC_{50} (Figure 4.1A). The parental cell line MDA-MB-468 was found to be less sensitive than in previous experiments, as cellular reductase activity was not decreased below 50% even at concentrations of 100μM CPX. The three remaining TNBC cell lines BT-20, Hs578t and HCC1937, were significantly less sensitive to the CPX-induced decrease in cellular reductase activity compared to the MDA-MB-231 or MDA16 TNBC cell lines (Figure 4.1A). The MDA-MB-231 cell line, similar to the MDA16 cell line, exhibits markers of clinically relevant MDR. These markers include amplification of the YWHAZ gene encoding for the anti-apoptotic protein 14-3-3ζ and amplification of the LAPTM4b gene encoding for a transmembrane efflux pump protein.

The non-TNBC cell lines tested, were significantly less sensitive than the MDA-MB-231 and MDA16 cell lines, as a 50% decrease in cellular reductase activity was only achieved at concentrations >10μM (Figure 4.1B). Both the colorectal and prostate cancer cell lines tested exhibited marked insensitivity to CPX. With the exception of the RKO colorectal and the DU145 prostate cancer cell lines, which had a 50% decrease in cellular reductase activity at 10μM, CPX failed to decrease cellular reductase activity below 50% in the remaining prostate and colorectal cancer cell lines at concentrations of 100μM (Figure 4.1C and D). The selectivity of CPX to decrease cellular reductase activity against cancer cell lines, was tested by performing a concentration response against the non-cancer breast cell line MCF10a. A 48 hour concentration response illustrated that CPX possesses an EC_{50} of approximately 25μM and is thus, almost 3 fold greater than the EC_{50} obtained in the MDA-MB-231 and MDA16 TNBC cell lines (Figure 4.1E).

Determining the molecular mechanism responsible for the effects of CPX in the sensitive breast cancer cell lines and the mode of cell death induced by CPX will be very useful for predicting how CPX may act in preclinical models of TNBC.

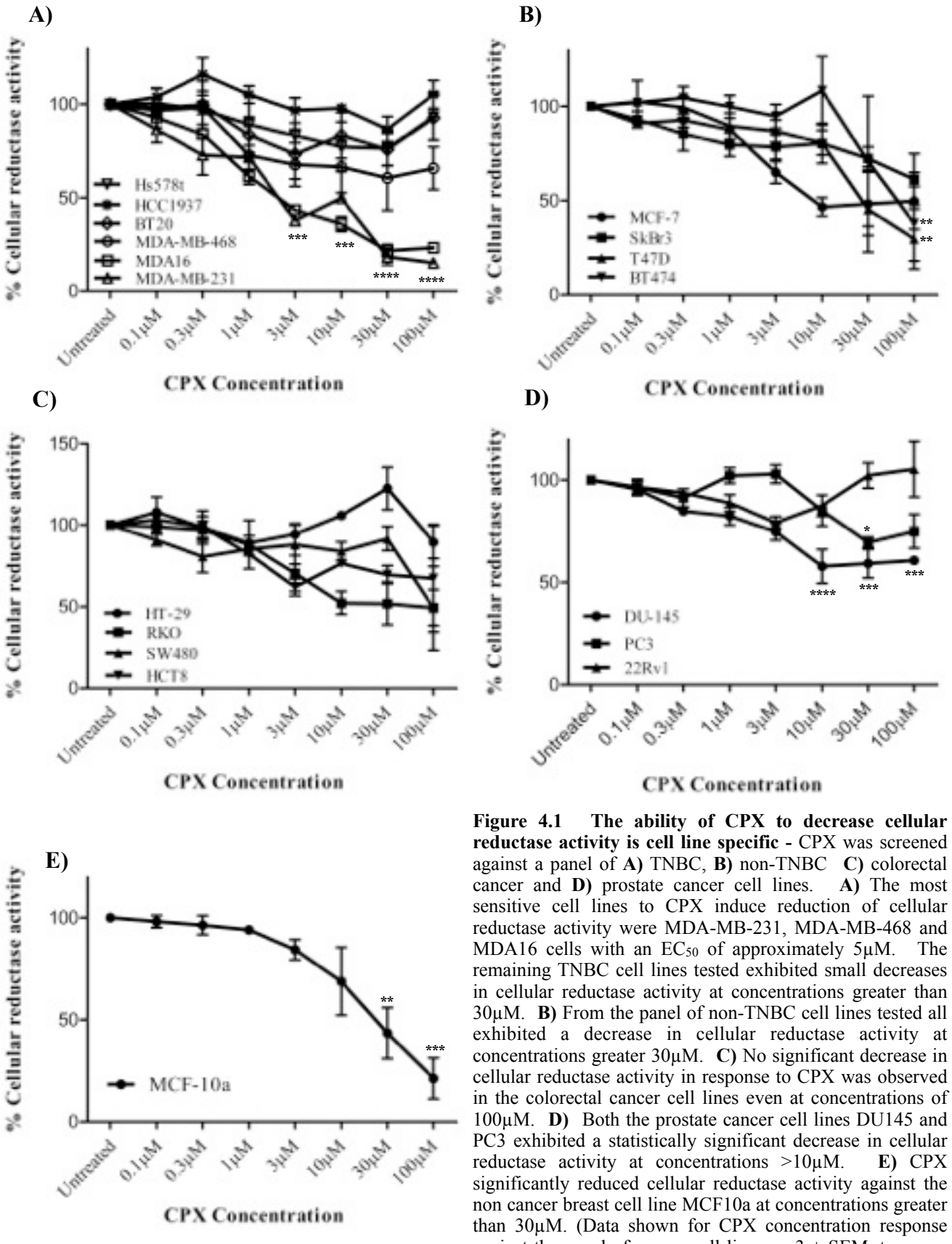


Figure 4.1 The ability of CPX to decrease cellular reductase activity is cell line specific - CPX was screened against a panel of **A)** TNBC, **B)** non-TNBC **C)** colorectal cancer and **D)** prostate cancer cell lines. **A)** The most sensitive cell lines to CPX induce reduction of cellular reductase activity were MDA-MB-231, MDA-MB-468 and MDA16 cells with an EC_{50} of approximately 5μM. The remaining TNBC cell lines tested exhibited small decreases in cellular reductase activity at concentrations greater than 30μM. **B)** From the panel of non-TNBC cell lines tested all exhibited a decrease in cellular reductase activity at concentrations greater 30μM. **C)** No significant decrease in cellular reductase activity in response to CPX was observed in the colorectal cancer cell lines even at concentrations of 100μM. **D)** Both the prostate cancer cell lines DU145 and PC3 exhibited a statistically significant decrease in cellular reductase activity at concentrations >10μM. **E)** CPX significantly reduced cellular reductase activity against the non cancer breast cell line MCF10a at concentrations greater than 30μM. (Data shown for CPX concentration response against the panel of cancer cell lines, n=3 ± SEM, two way ANOVA followed by Bonferroni post hoc test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Comparing the each concentration to untreated and the effect of each concentration between cell lines). (Data shown for the non-cancer breast cell line MCF10a is n=3, ±SEM one way ANOVA followed by Tukey's post hoc test. ** P<0.01, ***P<0.001, ****P<0.0001 (Comparing each concentration to untreated control).

4.2.2 *CPX induces apoptosis in sensitive TNBC cell lines*

In order to confirm the CPX-induced decrease in cellular reductase activity by the alamar blue assay, a number of complementary cell viability, cell number and apoptosis assays were performed on the MDA-MB-468 and MDA16 cell lines. Complementary viability assays were performed as one of the limitations with the alamar blue assay is that the cause of a decrease in cellular reductase activity cannot be deduced as it may be due to cell cycle arrest, senescence or cell death. Thus, the use of alternative assays will address this limitation. To assess if CPX affects plasma membrane integrity of the MDA-MB-468 cell line, a trypan blue assay was performed. Trypan blue is a negatively charged chromophore, that accumulates and stains the nuclei of cells with compromised plasma membrane integrity. The MDA-MB-468 cell line was treated with 10μM CPX and cells were incubated in the presence of CPX for 48 hours. After this time the MDA-MB-468 cell line was harvested and incubated for one minute in 0.4% trypan blue. Cells were counted and total cell number and viability was measured. CPX treatment induced a statistically significant, four fold reduction in cell number compared to DMSO vehicle control MDA-MB-468 cells (Figure 4.2A). There was a concurrent, statistically significant reduction in cell viability (Figure 4.2B).

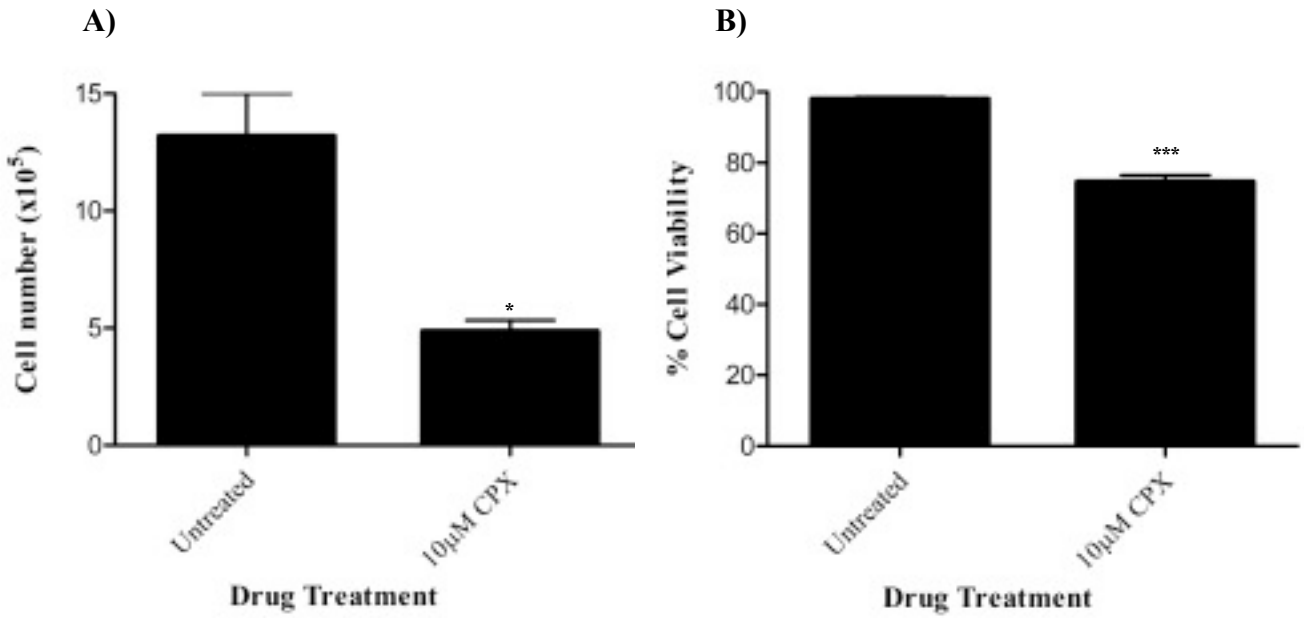


Figure 4.2 Treatment of MDA-MB-468 cells with CPX reduces both cell viability and number - MDA-MB-468 cells were treated with CPX for 48 hours, after this time cells were trypsinised and the trypan blue assay was performed. **A)** 48 hour CPX treatment induced a statistically significant reduction in cell number, ~ 4 fold, compared to untreated control in the MDA-MB-468 cell line, **B)** there was an associated and statistically significant decrease in cell viability. (Data shown n=3, ±SEM comparing drug treatment to untreated control, two-tailed t-test. * $P<0.05$, *** $P<0.001$).

One of the hallmarks of apoptotic cell death is maintenance of plasma membrane integrity. CPX treatment induced a four fold decrease in cell number compared to 48 hour untreated cells, but only a 20% decrease in cell viability as determined by the trypan blue assay. This suggests that CPX may be inducing cell cycle arrest, apoptosis or both in the MDA-MB-468 cell line. While characterising the mode of cell death induced by CPX, Zhou et al., 2010 reported that CPX induced apoptosis in TNBC cell line MDA-MB-231 (Zhou et al., 2010). To confirm that CPX induces apoptosis in the MDA-MB-468 and MDA16 cell lines; caspase-3 like activity, caspase processing and YO-PRO assays were performed. Activation of the apoptotic pathway induces cleavage of inactive caspase zymogens to their catalytically active forms, which in turn are capable of cleaving cellular proteins resulting in organised cellular dismantling. The MDA-MB-468 cell line was treated with either DMSO vehicle control, 10μM CPX or 1μM STS, (a well known inducer of caspase activity and processing). The MDA-MB-468 cell line was incubated in the respective treatments for 48 hours, after this time cells were harvested and lysed. A Bradford assay was performed on an aliquot of the lysed cells. The remaining sample was subjected to the caspase-3 like activity assay, which is measured using the synthetic caspase-3 fluorescent tetrapeptide substrate, DEVD-AFC. In the presence of active effector caspase-3/7, DEVD-AFC is cleaved resulting in increased fluorescence. MDA-MB-468 cells treated with 10μM CPX, exhibited an approximate seven fold increase in caspase-3 like activity compared to vehicle control (Figure 4.3A). The *bona fide* caspase activator staurosporine, also induced an approximate seven fold increase in fluorescence. The ability of CPX to induce caspase-9 and -3 processing was determined by immunoblotting. The MDA-MB-468 and MDA16 cell lines were treated with 10μM CPX and incubated in the presence of CPX for 48 hours, after this time cells were harvested, lysed, a bradford assay performed to determine protein concentration and samples were prepared for immunoblotting. Unprocessed caspase-9 exists as a 45kDa zymogen. Upon induction of an appropriate stress response such as genotoxic stress, pro-caspase-9 undergoes autocatalytic processing generating an active enzyme which activates the downstream effector caspase-3. Active caspase-3 in turn can cleave pro-caspase-9 generating a positive feedback, in addition to undergoing autocatalytic processing. CPX treatment of the MDA-MB-468 and MDA16 cell lines resulted in cleavage of caspase-9 shown by the appearance of the 35kDa and 37kDa fragments (Figure 4.3C), which is indicative of activation. Both MDA-MB-468 and MDA16 cell lines treated with 10μM CPX, exhibited processing of caspase-3 with generation of 19kDa and 17kDa fragments (Figure 4.3C) indicative of caspase activation. Active caspase-3 cleaves downstream cellular targets that result in dismantling of the cell.

The ability of 10µM CPX to induce caspase-mediated cleavage of the cellular substrate Lamin-B was tested. Treatment of the MDA-MB-468 cell line with 10µM CPX, resulted in the generation of the 45kDa lamin-B cleavage product (Figure 4.3B). This illustrates that CPX induces activation and processing of caspases, resulting in the cleavage of both synthetic and cellular caspase substrates.

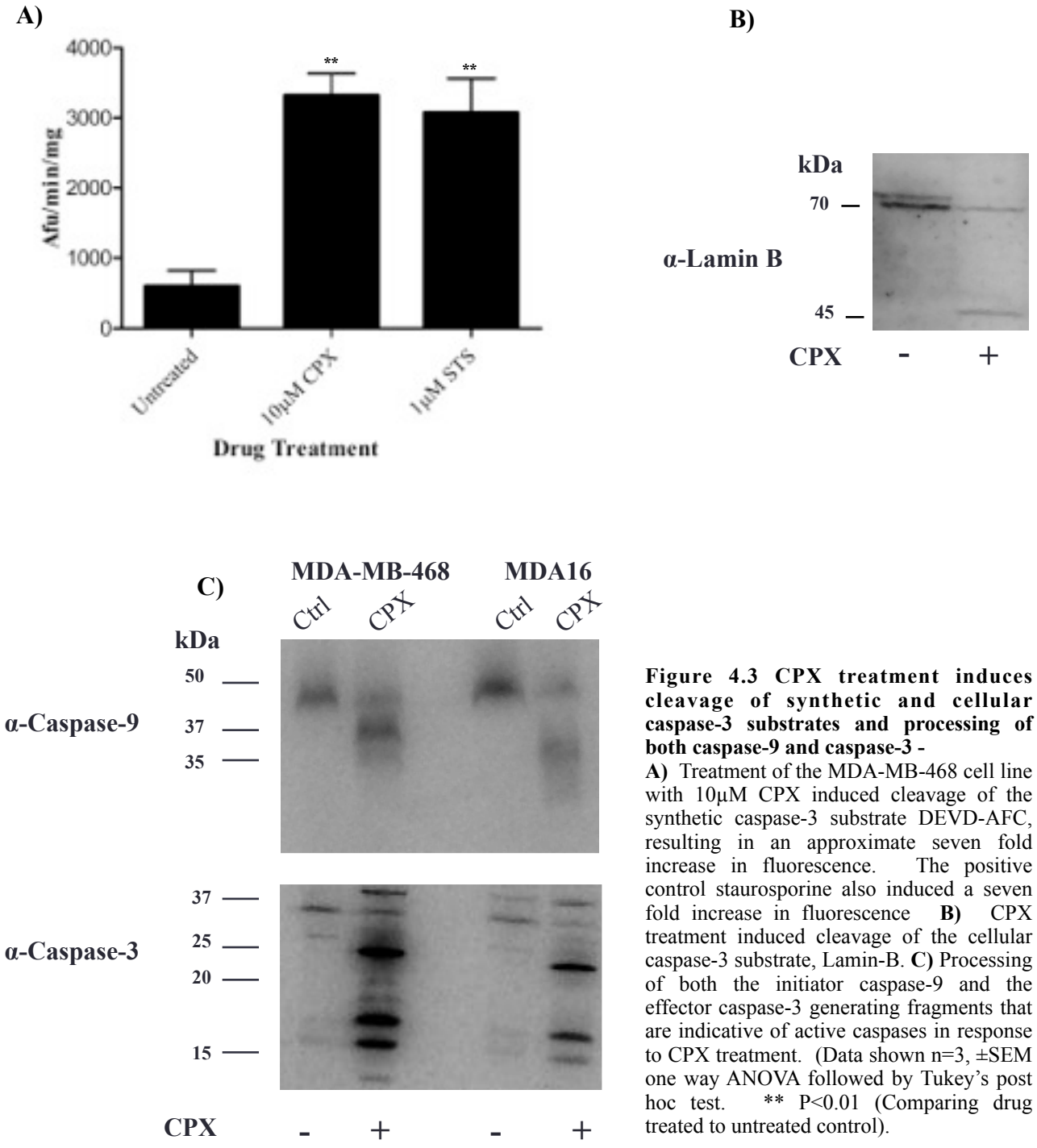


Figure 4.3 CPX treatment induces cleavage of synthetic and cellular caspase-3 substrates and processing of both caspase-9 and caspase-3 -
A) Treatment of the MDA-MB-468 cell line with 10µM CPX induced cleavage of the synthetic caspase-3 substrate DEVD-AFC, resulting in an approximate seven fold increase in fluorescence. The positive control staurosporine also induced a seven fold increase in fluorescence **B)** CPX treatment induced cleavage of the cellular caspase-3 substrate, Lamin-B. **C)** Processing of both the initiator caspase-9 and the effector caspase-3 generating fragments that are indicative of active caspases in response to CPX treatment. (Data shown n=3, ±SEM one way ANOVA followed by Tukey's post hoc test. ** $P<0.01$ (Comparing drug treated to untreated control).

YO-PRO is a fluorescent dye that enters and stains apoptotic cells (Idziorek et al., 1995). MDA16 cells were treated for 48 hours with 10µM CPX and cells were subsequently incubated in 250nM YO-PRO for 15 minutes to stain apoptotic cells. MDA16 cells were counterstained with propidium iodide, which stains DNA, to indicate the presence of cells that have undergone non-apoptotic cell death. A representative image from one experiment with CPX treated MDA16 cells can be seen in (Figure 4.4A and B). Baseline levels of approximately 2% YO-PRO positive and 4% PI/YO-PRO positive staining was identified in the DMSO vehicle control treated MDA-MB-468 cell line, indicating low levels of apoptosis under normal culture conditions (Figure 4.4C). The MDA16 cell line had slightly lower levels of basal apoptosis. Following treatment with 10µM CPX there was a 20% increase in YO-PRO positive staining in the MDA-MB-468 (Figure 4.4C) and a 30% increase in YO-PRO staining in the MDA16 cell line (Figure 4.4D), indicating the induction of apoptosis. There was a concurrent increase of YO-PRO/PI double positive staining in response to CPX treatment in the MDA-MB-468 cell line, approximately 20% (Figure 4.4D), and in the MDA16 cell line approximately 30% (Figure 4.4D), suggesting that cells have undergone either primary or secondary necrosis.

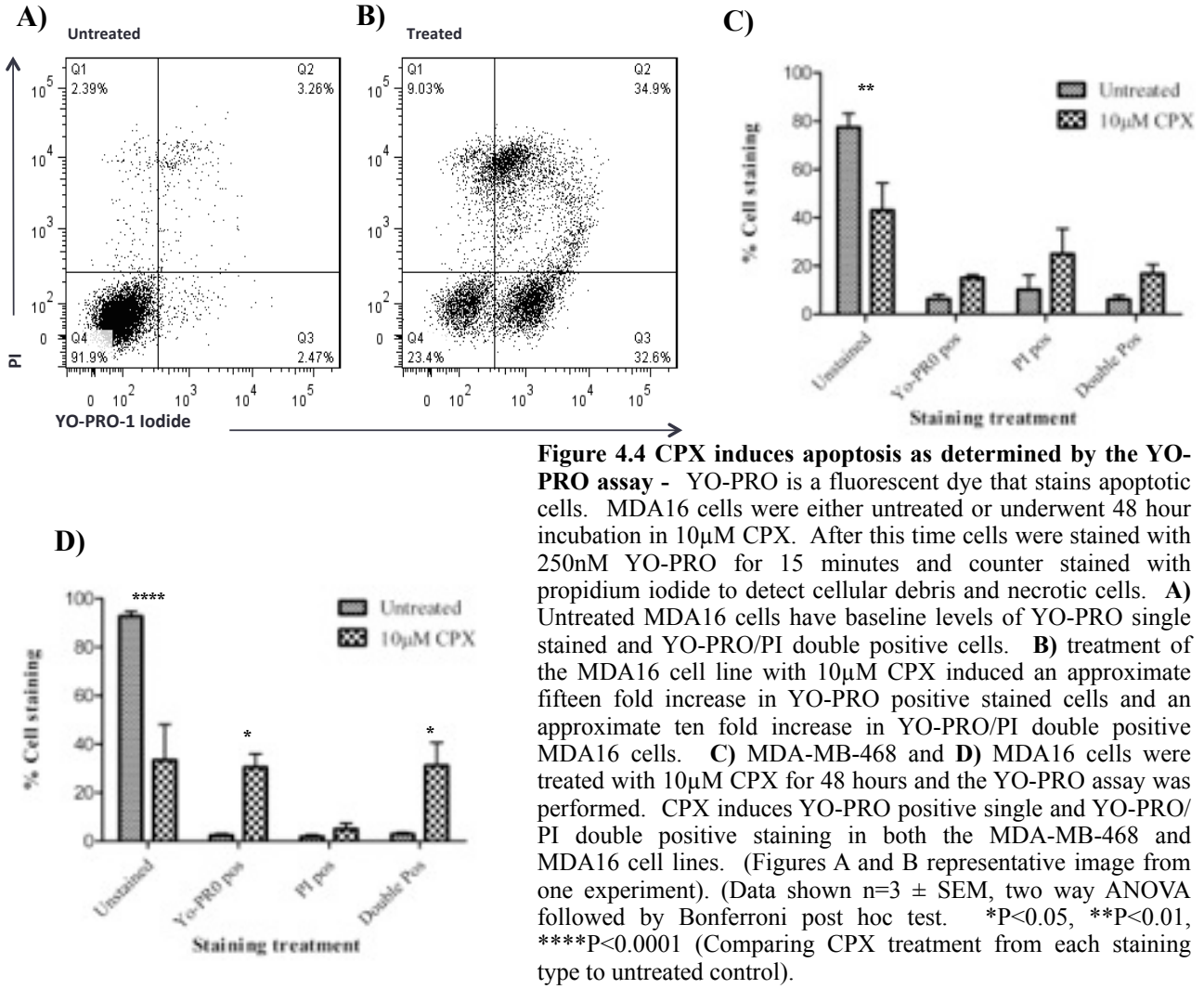


Figure 4.4 CPX induces apoptosis as determined by the YO-PRO assay - YO-PRO is a fluorescent dye that stains apoptotic cells. MDA16 cells were either untreated or underwent 48 hour incubation in 10µM CPX. After this time cells were stained with 250nM YO-PRO for 15 minutes and counter stained with propidium iodide to detect cellular debris and necrotic cells. **A)** Untreated MDA16 cells have baseline levels of YO-PRO single stained and YO-PRO/PI double positive cells. **B)** treatment of the MDA16 cell line with 10µM CPX induced an approximate fifteen fold increase in YO-PRO positive stained cells and an approximate ten fold increase in YO-PRO/PI double positive MDA16 cells. **C)** MDA-MB-468 and **D)** MDA16 cells were treated with 10µM CPX for 48 hours and the YO-PRO assay was performed. CPX induces YO-PRO positive single and YO-PRO/ PI double positive staining in both the MDA-MB-468 and MDA16 cell lines. (Figures A and B representative image from one experiment). (Data shown n=3 ± SEM, two way ANOVA followed by Bonferroni post hoc test. *P<0.05, **P<0.01, ****P<0.0001 (Comparing CPX treatment from each staining type to untreated control).

4.3 Discussion

4.3.1 CPX possesses cytotoxic activities against cancer cell lines and in preclinical models of cancer

CPX was validated from the secondary screen as one of the most effective, non-neoplastic compounds at decreasing cellular reductase activity. CPX is currently approved for the treatment of topical mycoses, such as fungal skin infections and vaginal candidiasis. In addition, CPX is currently being tested in clinical trials against a range of other superficial bacterial and fungal diseases and information relating to the particular diseases, design and proposed end date of these trials can be found on the clinicaltrial.gov website.

Coincident with our identification of CPX as a compound that decreased cellular reductase activity in the MDA16 cell line, data showing that CPX possessed cytotoxic activities *in vitro* against a panel of leukemic cancer cell lines and decreased the rate of tumour growth in preclinical xenograft models of leukemia was reported (Eberhard et al., 2009). CPX was identified by screening a library containing off-patent drugs against a panel of leukemic and multiple myeloma cancer cell lines *in vitro* (Eberhard et al., 2009). After establishing that CPX possessed cytotoxic activities against leukemia and multiple myeloma cancer cell lines, the *in vivo* efficacy of CPX was tested. CPX was tested against three preclinical leukemic xenograft models and identified to significantly decrease tumour weight, indicating antitumour properties (Eberhard et al., 2009). These promising *in vitro* and preclinical leukemic xenograft model results, led to the designing of a clinical trial assessing the potential anticancer activity of CPX against a broad range of haematological malignancies in humans. The reported end data and primary outcome measures of this clinical trial are expected in October 2012 (University Health Network, 2011). Following identification of the cytotoxic activities associated with CPX against leukemic cancer cell lines, a second paper was published reporting that CPX has cytotoxic activities against the TNBC cell line MDA-MB-231 (Zhou et al., 2010). CPX was shown to significantly decrease tumour growth in a preclinical breast cancer xenograft model and in addition, the mode of cell death induced by CPX was identified as apoptosis both *in vitro* and *in vivo* (Zhou et al., 2010)

Further characterisation of CPX, subsequent to its initial identification from the JHCCL in the primary screen, identified that a subset of TNBC cell lines, MDA-MB-468, MDA-MB-231 and MDA16, were sensitive to CPX. Interestingly, there are a subset of TNBC cell lines that did not respond to CPX even at concentrations of 100μM. While some of the non-TNBC, colorectal cancer and prostate cancer cell lines did exhibit decreases in cellular reductase activity, the EC_{50} was between 10-90μM greater than the EC_{50} against the CPX sensitive TNBC cell lines.

Thus, it is promising that CPX displays selectivity for a subset of the TNBC cell lines tested and did not significantly decrease cellular reductase activity of the non-cancer breast cell line MCF10a within the concentration required for its activity against the sensitive TNBC cell lines. Determining the factors that contribute to CPX sensitivity in the subset of TNBC cell lines, will provide useful information for human breast cancers, that are most likely to respond positively to CPX if it is to be successfully repurposed as an anticancer therapy.

4.3.2 Apoptosis is the primary mode of cell death induced by CPX in both MDA-MB-468 and MDA16 TNBC cell lines

The primary mode of cell death induced in MDA-MB-468 and MDA16 TNBC cell lines by CPX is apoptosis. This was confirmed following identification of increased YO-PRO staining, caspase-3 like activity, cleavage of both caspase-9 and -3 and cleavage of the cellular caspase substrate lamin-B, following 48 hour incubation in 10μM CPX. These findings correlate with published data reporting that CPX treatment decreased cell number and increased caspase-3 like activity in the TNBC cell line MDA-MB-231. Both the decrease in MDA-MB-231 cell number and induction of caspase-3 like activity was inhibited following co-treatment of CPX with the fluorescent caspase inhibitor z-VAD-FMK (Zhou et al., 2010). CPX has been shown to induce a statistically significant decrease in the growth of a MDA-MB-231 breast cancer xenograft model. MDA-MB-231 tumours removed from the xenograft model were shown to have increased TUNEL positive staining (Zhou et al., 2010). Identifying and characterising the mode of cell death, provides interesting information relating to how sensitive cells interpret and respond to the stress induced by CPX. However, this information does not inform or clarify the cellular target(s) of CPX that are responsible for its cytotoxicity.

Thus, identification of the cellular target(s) responsible for CPX mediated cytotoxicity, will provide invaluable information regarding the types of cancers that will most likely respond to CPX treatment and will also allow for the stratification of patients based on those that are most likely to respond favourably to CPX based intervention.

4.3.3 Iron chelation appears to be CPX's mechanism of action

Following the identification that CPX possessed cytotoxic activities against leukemic and multiple myeloma cancer cell lines and decreased tumour weight in preclinical leukemic xenograft models, Eberhard et al.,2009 attempted to identify the mechanism of action responsible for CPX's activity (Eberhard et al., 2009). CPX was shown to bind intracellular iron and this effect was illustrated to be functionally important as supplementing medium with iron protected cancer cells from CPX induced decreased viability (Eberhard et al., 2009). A literature search of CPX, molecular targets and cancer generates a large list of proteins whose expression in response to CPX treatment is effected. Whether this change in protein expression is a cause or a consequence of CPX treatment has not been well established to date. Below are a few paragraphs of some proteins that have been tested in response to CPX treatment and have been shown to respond to CPX due to its ability chelate iron.

For over two decades it has been reported that CPX induces cell cycle arrest in human cancer cell lines (Hoffman et al., 1991). Indeed CPX and related iron chelators have been used experimentally to synchronise a population of cells to the same phase of the cell cycle. CPX is a well established iron chelator and recent research suggests that its ability to inhibit the cell cycle during the initiation phase of DNA replication is associated with the induction of DNA double strand breaks and activation of DNA damage response signalling. This DNA damage induced by CPX is reportedly attributable to its iron chelation abilities (Szuts and Krude, 2004). The enzyme ribonucleotide reductase (RNR) plays a central role in DNA synthesis by converting nucleoside diphosphates into deoxynucleoside diphosphates thereby maintaining a pool of deoxyribonucleotides for DNA synthesis. In order to function, RNR requires iron and it has been shown that CPX inhibits RNR activity (Eberhard et al., 2009).

The eukaryotic translation initiation factor (eIF5a-2) protein plays a critical role in protein translation. eIF5a-2 is unique, as it is believed to be the only protein containing the modified amino acid hypusine, which is formed by the post-translational modification of a lysine residue. This process is catalysed sequentially by the enzymes deoxyhypusine synthase and deoxyhypusine hydroxylase. Functional eIF5a-2, is critically dependent on the presence of the hypusine (Hoque et al., 2009). The eIF5a-2 gene is located on the gene locus 3q26 which is reportedly amplified in some hepatocellular carcinoma, lung and ovarian cancers (Guan et al., 2004, He et al., 2011, Tang et al., 2010). Amplification of the 3q26 gene locus is a reported marker of poor prognosis in patients with lung cancer. CPX has been shown to inhibit the activity of deoxyhypusine hydroxylase *in vitro* and this inhibition is associated with decreased proliferation and motility rates in cancer cell lines known to have over-expression of eIF5a-2 (Tang et al., 2010).

Hypoxia induced factor-1α (HIF-1α), is a transcription factor that plays a critical role in maintaining cellular homeostasis in response to low oxygen levels. Under such hypoxic conditions, HIF-1α is stabilised by oxygen and iron dependent hydroxylation of proline and asparagine residues on the α-subunit. This stabilisation process allows for the transcription of HIF-1α target genes, such as the VEGF receptor and glycolytic enzymes, thereby allowing the cell to adapt to decreased oxygen conditions (Linden et al., 2003). It been suggested that pharmacological inhibition of HIF-1α may be a novel anticancer approach, as over-expression of HIF-1α is associated with advanced disease, poor prognosis and development of resistance among cancer patients. Interestingly, HIF-1α expression is stabilised under normoxic conditions in response to CPX treatment (Nagle and Zhou, 2006). If the mode of action of HIF-1α under normoxic conditions, is the same as the mode of action under hypoxic conditions, it could be assumed that stabilisation of HIF-1α and expression of its target genes is likely to benefit the growth of the cancer cell/tumour. The up-regulation of VEGF receptor and induction of glycolytic enzymes are likely to increase the growth and potential metastasis of tumour cells (Unruh et al., 2003).

Iron is a common and essential co-factor required for the activity of numerous cellular proteins including enzymes. All of the aforementioned targets are responsive to the effects of CPX due to its ability to chelate iron and thus, interfere with iron dependent cellular process. Due to the recent identification that CPX may possess novel anticancer activities and the need to identify a critical target(s) responsible for CPX mediated cytotoxicity, it is likely that the repertoire of CPX targets will increase in the future.

However, the cytotoxic activities of CPX *in vitro* and in preclinical breast and leukemic xenograft models, may require CPX to effect a number of key processes whose components require iron as a co-factor. Thus, the difficultly faced in successfully repurposing CPX as an anticancer agent, is determining what predisposes a cancer cells sensitivity to CPX. Identifying this, will allow for the stratification of patient tumours likely to respond to CPX. It has been observed that haematological cancers, particularly disseminated in nature, have a higher growth fraction compared to solid tumours, as their growth rate is not limited by passive diffusion of oxygen and growth factors to sustain growth. As a result, haematological cancers are inherently more sensitive to inhibitors of the cell cycle and DNA damaging agents compared to solid tumours, such as breast cancers which do rely on passive diffusion (Friberg and Mattson, 1997, Hobbs, 1969). Thus, without identifying a molecular marker(s) indicative of responsiveness to CPX, it will be difficult for CPX to prove successful in the treatment of solid tumours.

4.3.4 CPX's pharmacokinetics and toxicology

Despite the current lack of cellular targets known to be responsible for the cytotoxic activity of CPX in cancer cell lines, two clinical trials are currently underway testing the potential anticancer activity of CPX. The first trial is testing the anticancer activity of orally administered CPX against a range of haematological malignancies. The pharmacokinetics of systemically administered CPX is not well known in humans. Indeed only one study using orally administered CPX has been conducted with healthy humans. A single dose of 10 mg/kg was administered, after 12 hours 96% of the dose was recovered in the urine metabolised via glucuronidation and no toxicity issues were reported with the single dose. Due to the lack of pharmacological and toxicological data relating to systemically administered CPX, data obtained using preclinical studies in dogs, rats and mice have been extrapolated. A dose related increase in myocardial degeneration was observed following repeated dosing with oral CPX in rats and dogs. This observation suggests, patients with existing heart conditions should be closely monitored for any exacerbation of the preexisting condition. Additionally, the co-treatment of CPX with chemotherapeutics known to induce cardiotoxicity such as doxorubicin or trastuzumab, may result in additive or synergistic toxicity issues. Cardiac toxicity, liver and lung degeneration was also reported (Weir et al., 2011). Despite these adverse effects, the dose associated with toxicity is higher than the dose required for efficacy in leukemia and breast cancer xenograft models (Eberhard et al., 2009, Zhou et al., 2010).

The second clinical trial is testing the effect of topical CPX as a prophylactic treatment against precancerous lesions of the lower female genital tract (Cracchiolo, 2006, Minden, 2011). As a topical agent, there exists a significantly greater pharmacokinetic and toxicological profile in humans, as this is the primary formulation of CPX when used as an antifungal agent. Following application of 1% CPX cream, 1.3% of the dose was absorbed with total penetration occurring after 6 hours. Excretion occurs via the kidneys in the same manner as systemically administered CPX and the drug is inactivated via glucuronidation. Toxicity issues following topical administration include local burning sensation, headache, rash, local irritation and dry skin but are significantly less life threatening than high oral dose CPX (Weir et al., 2011).

Thus, while not much data is reported regarding systemic administration of CPX in humans, extrapolation of the data from preclinical studies suggests that pharmacologically relevant concentrations of CPX can be achieved. However, CPX is rapidly cleared from the plasma, therefore it seems likely that repeated daily doses may be required to maintain therapeutically relevant concentrations in the body sufficient for anticancer activity. Results from the clinical trail of repeated orally administered CPX, for the treatment of leukemic cancers will provide invaluable information relating to achievable concentrations in humans and potential toxicity issues.

Due to CPX affecting numerous cellular targets, at present it is not known whether these changes are a cause of consequence of CPX treatment. The ramification of this is that without identifying a molecular target that is causally responsible for CPX cytotoxicity in cancer cells, or indeed a biomarker of sensitivity increases the difficulty in repurposing CPX as a potential anticancer agent. To further compound this CPX is well reported to possess a poor pharmacokinetic profile. In light of this evidence CPX was not selected for further characterisation, instead the antibiotic ANS was selected.

Chapter 5

Characterising the activities of the antibiotic anisomycin against cancer cell lines *in vitro*

5.1 Introduction

ANS is produced by the bacterium *Streptomyces griseolus* and was clinically approved as an antiprotozoal for the treatment of intestinal amebiasis (Macias-Silva, 2010); original articles [(Constandse, 1956, Seneca, 1955)]. From the mid 1960's, clinical literature on ANS is almost non-existent and it appears that ANS's primary use was as a laboratory research tool. ANS is most well known for its ability to bind the 60S subunit of functional 80S ribosome and thereby inhibit the enzyme peptidyl transferase. Indeed, the crystal structure of ribosome-bound ANS has been elucidated (Hansen et al., 2003, Bulkley et al., 2010). Peptidyl transferase catalyses the formation of peptide bonds between adjacent amino acids bound to tRNA. Blocking peptidyl transferase activity ultimately results in the inhibition of protein synthesis (Caskey et al., 1971, Iordanov et al., 1997, Barbacid and Vazquez, 1975). Only one molecule of ANS binds per ribosome and the affinity of ANS to the 60S subunit of functional 80S ribosomes is very strong with a K_d of $1.6x10^{-6}$ being determined using human tonsil ribosomes (Barbacid and Vazquez, 1974). Inhibition of protein synthesis by ANS induces phosphorylation and activation of the stress activated kinases JNK and p38 (Iordanov et al., 1997). Induction of these kinases in some cell lines, has been associated with activation of caspases and subsequent cell death by apoptosis (Weston and Davis, 2007, Ruller et al., 1999). The process of protein synthesis inhibition, with induction of stress signalling via JNK and p38, with subsequent activation of apoptotic cell death is referred to as "ribotoxic stress" (Iordanov et al., 1997, Ouyang et al., 2005).

ANS has been reported to possesses cytotoxic activities against cancer cell lines *in vitro* and in preclinical syngenic tumour models. ANS has been shown to sensitise the prostate cancer cell line DU145 and the human glioblastoma cancer cell line U87 to the death receptor ligand Fas or Fas and TRAIL mediated apoptosis (Xia et al., 2007, Curtin and Cotter, 2002). The ability of ANS to sensitise both the DU145 and U87 cancer cell lines to apoptosis is reportedly due to ANS mediated induction of JNK phosphorylation (Xia et al., 2007, Curtin and Cotter, 2002). Despite the ability of ANS to sensitise prostate and glioblastoma cancer cell lines, the dependency on JNK for sensitisation has only been clearly established in the U87 glioblastoma cell line. In this experiment, pharmacological inhibition of JNK followed by co-treatment with ANS and Fas protected the U87 glioblastoma cancer cell line from ANS-induced sensitisation and induction of apoptosis (Xia et al., 2007). This was not shown in the sensitisation of the DU-145 prostate cancer cell line to Fas by ANS.

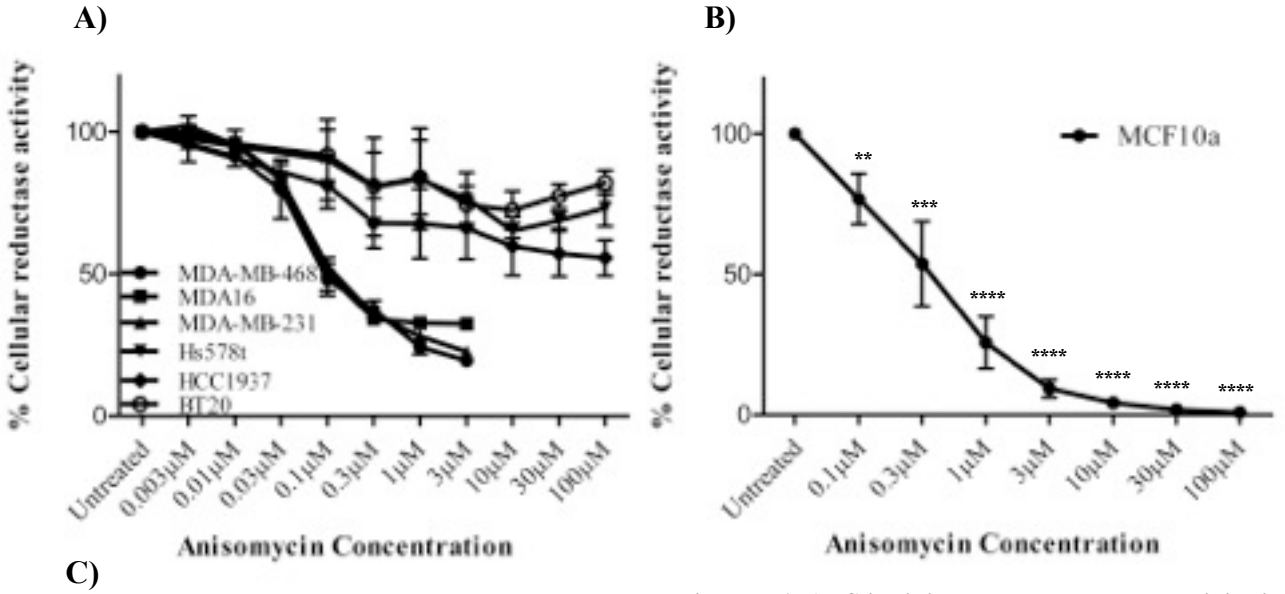
Interestingly, ANS has been shown to sensitise resistant cancer cells to anoikis and this was due to ANS inhibiting protein synthesis and causally decreasing the expression of the short half-life anti-apoptotic protein, c-FLIP (Mawji et al., 2007). c-FLIP negatively regulates formation of the death receptor complex. Co-treatment of ANS with Fas in the DU145 prostate cancer cell line, may induce cell death via the death receptor due to ANS decreasing c-FLIP expression and propagation of a death signal by Fas. Despite being suggested as an alternative explanation this was not tested (Curtin and Cotter, 2002). More recently it has been reported that ANS decrease cellular reductase activity with an EC_{50} of approximately 6.5μM against the MCF-7 and BT-474 breast cancer cell lines *in vitro* (Liu et al., 2009). However, the exact mode of cell death induced by ANS in these human breast cancer cell lines was not established. ANS was found to induced caspase-3 activation in the mouse mammary cancer cell line TSA *in vitro* and significantly reduce tumour growth rate in a preclinical syngenic mouse model, using the mouse mammary cancer cell line TSA (Liu et al., 2009). This suggests that ANS may induce apoptosis in the TSA cell line which contributes to the decrease in tumour growth rate. The aim of this study was to identify the spectrum of ANS's activity, by screening against a panel of TNBC, non-TNBC, colorectal and prostate cancer cell lines. To determine if ANS induces ribotoxic stress as reported in the literature and if ribotoxic stress is required for ANS induced cell death. As outlined the only reported mode of cell death induced by ANS is caspase-dependent apoptosis, thus, in the present study the potential role and significance of caspase activation using the TNBC cell line MDA-MB-468 in response to ANS was tested.

5.2 Results

5.2.1 ANS effectively decreases both cellular reductase activity and cell number in a subset of cancer cell lines

ANS was tested against a panel of cancer cell lines. An ANS concentration response was performed as outlined in (section 2.2.1) and cells were incubated in the presence of the drug for 48 hours. After this incubation step, alamar blue was added and cells were subsequently incubated for a further six hours before fluorescence was measured. From the panel of TNBC cell lines subjected to an ANS concentration response, a subset of the TNBC cell lines are more sensitive to ANS-mediated decrease in cellular reductase activity (Figure 5.1A).

ANS induced a statistically significant decrease in the cellular reductase activity of the MDA-MB-468, MDA-MB-231 and MDA16 TNBC cell lines, with an EC_{50} of 100nM (Figure 5.1A). The remaining TNBC cell lines were significantly less responsive to the ANS-induced decrease in cellular reductase activity. Only the HCC1937 cell line exhibited a statistically significant decrease in cellular reductase activity with a 50% decrease at concentrations >30μM (Figure 5.1A). ANS induced a statistically significant decrease in cellular reductase activity with an EC_{50} of approximately 300nM against the non-cancer breast cell line MCF10a (Figure 5.1B), a concentration comparable to the pharmacologically relevant concentration responsible for the decrease in cellular reductase activity observed in the ANS sensitive TNBC cell lines. The relative decreases in cellular reductase activity induced by 1μM ANS against the panel of TNBC cell lines and the non-breast cancer cell lines MCF10a is illustrated in (Figure 5.1C).



C)

Cell Line	% Cellular Reductase activity at 1μM (±SEM)	Statistical significance
MDA-MB-468	24.27 ± 2.57	P<0.0001
MDA16	32.67 ± 1.48	P<0.0001
MDA-MB-231	28.1 ± 0.42	P<0.0001
BT20	83.98 ± 13.10	NS
Hs578t	83.47 ± 17.80	NS
HCC1937	67.75 ± 12.31	NS
MCF10a	25.5±2.5	P<0.0001

Figure 5.1 ANS inhibits cellular reductase activity in a subset of TNBC cell lines - An ANS concentration response was performed against a panel of TNBC cell lines. Cells were incubated in the presence of ANS for 48 hours and after this time the alamar blue assay was performed. **A)** Anisomycin is selectivity more effect at decreasing cellular reductase activity against a subset of TNBC cell lines, **B)** ANS effectively decrease cellular reductase activity in the non-cancer breast cell line MCF10a, **C)** Table representing the data from (Figure A) illustrating the % cellular reductase in response to ANS, on the TNBC cell lines tested. (Data shown for ANS concentration response against the panel of TNBC cell lines is n=3 ± SEM, two way ANOVA followed by Bonferroni post hoc test, *P<0.05, **P<0.01, ****P<0.0001 (Comparing the effects of ANS to both untreated control and the effect of ANS between cell lines). (Data shown for the ANS concentration response against the non-cancer breast cell line is n=3, ±SEM one way ANOVA followed by Tukey's post hoc test. ** P<0.01, ***P<0.001)

The non-TNBC cell lines tested, displayed insensitivity towards ANS, with no statistically significant decrease in cellular reductase activity detected even at concentration of 100μM (Figure 5.2A). The panel of colorectal cancer cell lines tested were also highly insensitive to the effects of ANS, with no significant decrease in cellular reductase activity evidenced at any concentration (Figure 5.2B). ANS induced a statistically significant decrease in cellular reductase activity against the prostate cancer cell lines tested. The most sensitive prostate cancer cell line to ANS-mediated decrease in cellular reductase activity was the androgen insensitive DU145 cell line, against which ANS possesses an EC_{50} of 500nM. ANS effectively decreased cellular reductase activity of another androgen insensitive prostate cancer cell line 22Rv1, with an EC_{50} of approximately 1μM (Figure 5.2C). The final prostate cancer cell line tested was the PC3 cell line. The PC3 cell line was found to be less sensitive to ANS-mediated decrease in cellular reductase activity compared to the 22Rv1 and DU145 prostate cancer cell lines. ANS did induce a statistically significant decrease in cellular reductase activity in the PC3 cell line, but even at concentrations of 100μM, ANS failed to induce a 50% decrease in cellular reductase activity after 48 hours.

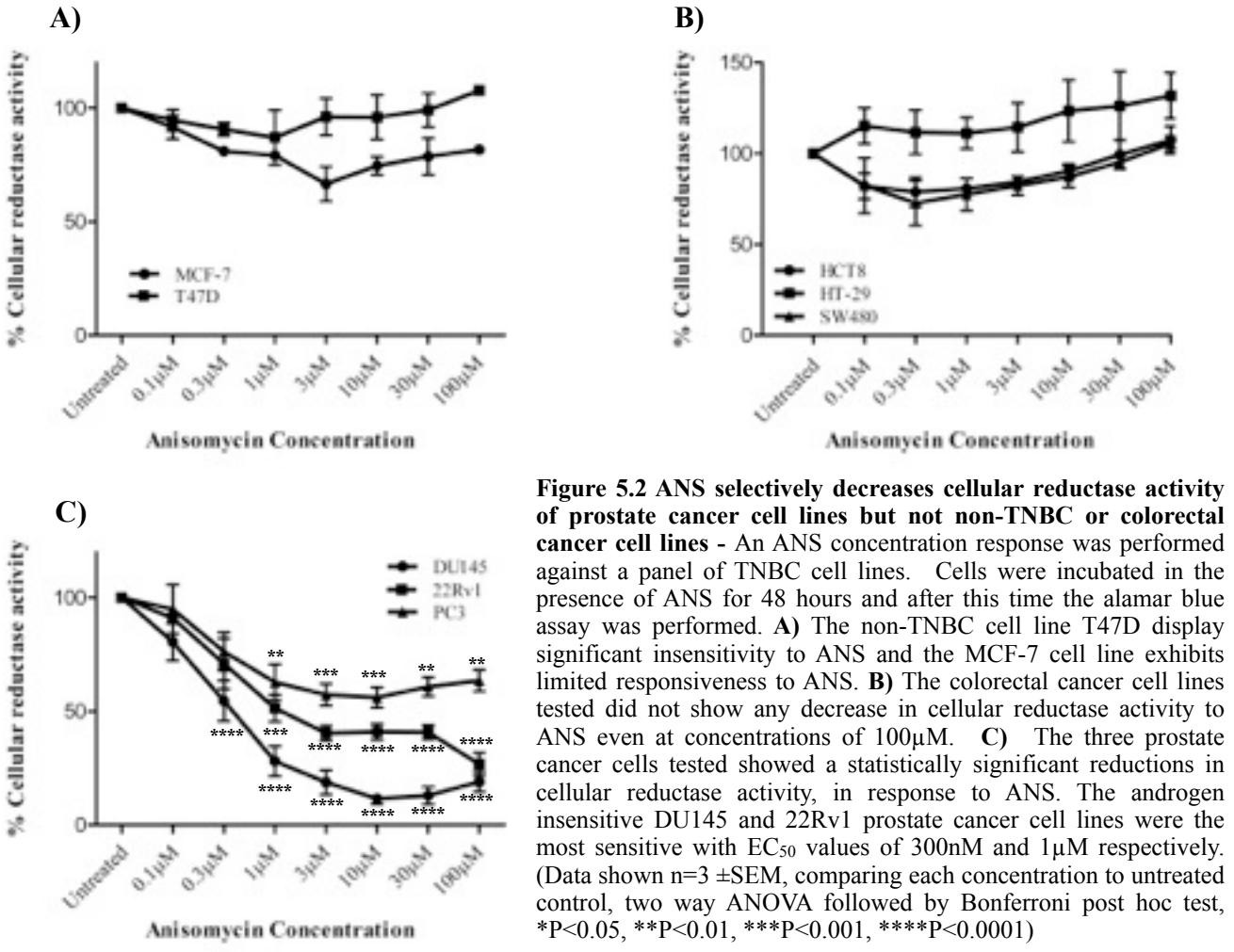


Figure 5.2 ANS selectively decreases cellular reductase activity of prostate cancer cell lines but not non-TNBC or colorectal cancer cell lines - An ANS concentration response was performed against a panel of TNBC cell lines. Cells were incubated in the presence of ANS for 48 hours and after this time the alamar blue assay was performed. **A)** The non-TNBC cell line T47D display significant insensitivity to ANS and the MCF-7 cell line exhibits limited responsiveness to ANS. **B)** The colorectal cancer cell lines tested did not show any decrease in cellular reductase activity to ANS even at concentrations of 100μM. **C)** The three prostate cancer cells tested showed a statistically significant reductions in cellular reductase activity, in response to ANS. The androgen insensitive DU145 and 22Rv1 prostate cancer cell lines were the most sensitive with EC_{50} values of 300nM and 1μM respectively. (Data shown n=3 ±SEM, comparing each concentration to untreated control, two way ANOVA followed by Bonferroni post hoc test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

ANS is known to induce apoptosis *in vitro,* thus in an attempt to determine the mode of cell death induced by ANS in the present study, a number of cell viability and cell death assays were performed. The MDA-MB-468 TNBC cell line was treated with 1μM ANS and cells were incubated in the presence of ANS for 48 hours. After this time both untreated control and 1μM ANS treated MDA-MB-468 cells were harvested and a cell count was performed. Treatment of the MDA-MB-468 cell line for 48 hours with 1μM ANS, induced a statistically significant decrease in cell number compared to the untreated control (Figure 5.3)

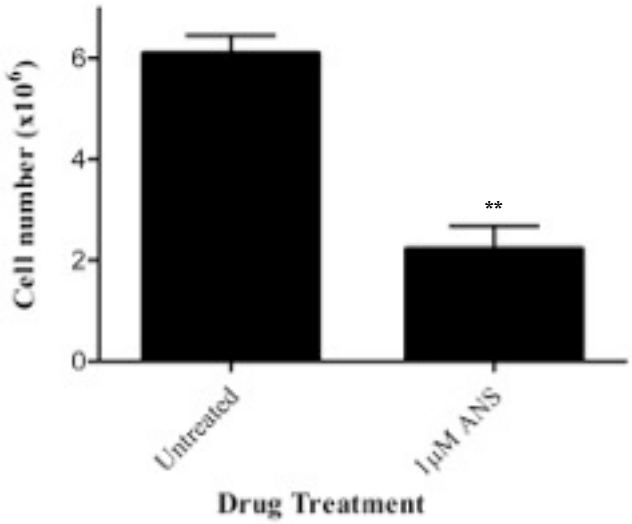
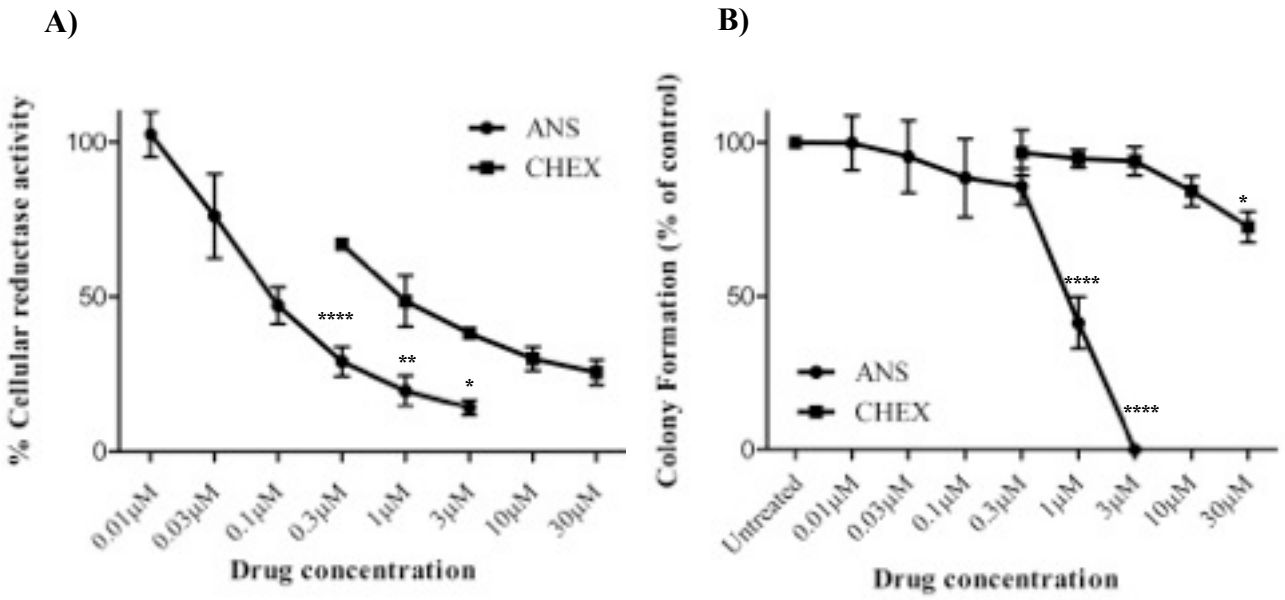


Figure 5.3 ANS decreases cell number in the MDA-MB-468 cell line - The MDA-MB-468 cell line was treated with 1μM ANS and incubated in the drug for 48 hours, after this time a cell count was performed. 1μM ANS induced a statistically significant three fold decrease in cell number. (Data shown comparing 1μM ANS to untreated control, n=3 ± SEM, significance determined using the t-test. **P<0.01)

5.2.2 ANS is more effective at decreasing colony formation than the protein synthesis inhibitor cycloheximide

ANS binds to the 60S subunit of functional 80S ribosomes, inhibits peptidyl transferase activity and thereby shuts down protein synthesis in cells (Barbacid and Vazquez, 1975, Janssen, 1977). Cycloheximide (CHX) also inhibits protein synthesis but does so by preventing the release of deacylated tRNA thus, inhibiting the translocation of peptidyl-tRNA from the A to P site of actively translating ribosomes (Verbin, 1967, Grollman, 1966). To test if the MDA-MB-468 cell line was sensitive to another protein synthesis inhibitor, a 48 hour ANS and CHX concentration response was performed. The ability of each compound to decrease cellular reductase activity and their effects on colony formation were tested. Both ANS and CHX were found to induce similar decreases in cellular reductase activity in the MDA-MB-468 cell line. ANS induced a statistically significant decrease in cellular reductase activity, possessing an EC_{50} of approximately 100nM as previously shown. CHX also induced a statistically significant decrease in cellular reductase activity, with an EC_{50} of approximately 1μM (Figure 5.4A). Thus, there is approximately a ten fold difference between the effectiveness of ANS and CHX at decreasing cellular reductase activity, but the effects are comparable. Next, the ability of ANS and CHX to effect colony formation was tested by incubating the MDA-MB-468 cell line in a range of concentrations of both compounds for 24 hours. After this time-point the medium containing drug was removed, cells were washed with fresh medium and subsequently incubated in fresh medium for one week. At the end of the experiment cells were washed, fixed and incubated in crystal violet dye for 10 minutes to stain colonies and the number of colonies were counted. Treatment of the MDA-MB-468 cell line with 1μM ANS induced a statistically significant 50% decrease in the number of colonies and 3μM ANS completely inhibited the formation of all colonies. In contrast, CHX induced a statistically significant decrease in colony formation only at a concentration of 30μM, where the number of colonies were reduced by only 25% (Figure 5.4B). Thus, despite inducing similar decreases in cellular reductase activity, the ability of ANS and CHX to inhibit colony formation were significantly different.

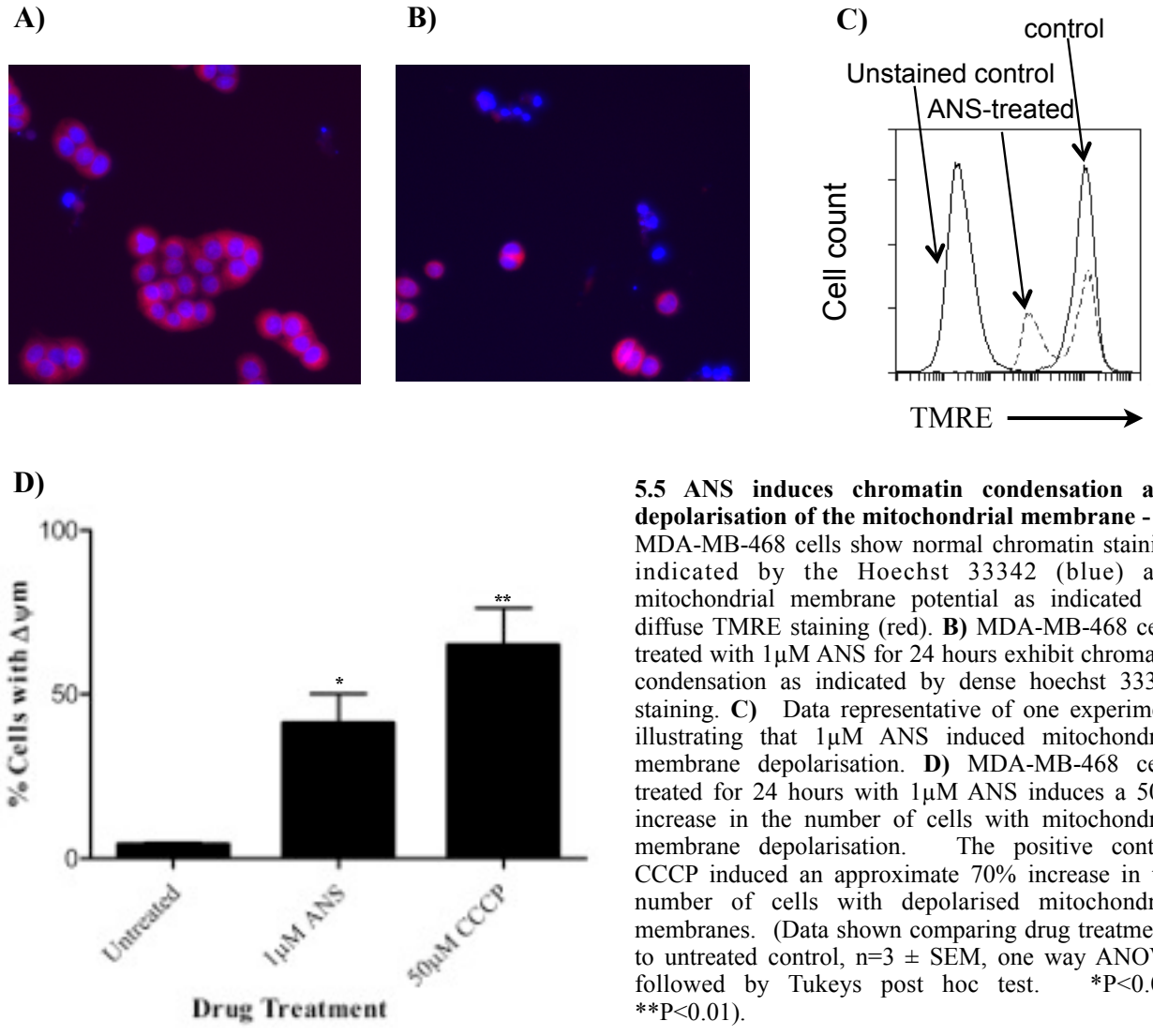


5.4 The protein synthesis inhibitor CHX is not as effective at decreasing colony formation as ANS - A) The MDA-MB-468 cell line was treated with a concentration response of either ANS or CHX and incubated for 48 hours. Both ANS and CHX have comparable effectiveness in their ability to decrease cellular reductase activity. **B)** The MDA-MB-468 cell line was seeded at a concentration of 30,000 cells per T25 and a CHX/ANS concentration response performed. Cells were incubated for 24 hours with each treatment, after this time cells were washed and fresh medium added. 1μM ANS induces a 50% decrease in the number of colonies and 3μM ANS induced a 100% inhibition of colony formation. CHX induces a statistically significant decrease in the the number of colonies with an approximate 20% decrease in colony number at a concentration of 30μM. (Data shown n=3, ± SEM, comparing each concentration to untreated control, two way ANOVA followed by a Bonferroni post hoc test. *P<0.05, ****P<0.0001).

5.2.3 *ANS induces chromatin condensation and loss of mitochondrial membrane potential in the MDA-MB-468 cell line*

Chromatin condensation is a major morphological hallmark used to characterise cells that are undergoing apoptotic cell death. ANS has been reported to induce caspase activation, which is essential for cell death in certain cancer cell lines. Thus, the ability of ANS to induce chromatin condensation in the MDA-MB-468 cell line was tested. MDA-MB-468 cells were treated with 1μM ANS for 24 hours, after this time both untreated control and ANS treated cells were stained with the mitochondrial dye TMRE (red) and the nuclear dye Hoechst 33342 (blue) for fifteen minutes and cells were subsequently visualised using a fluorescent microscope. Normal mitochondria with polarised membranes will readily accumulate TMRE and stain red. Abnormal mitochondria with a depolarised membrane potential will not accumulate TMRE resulting in diffuse cytoplasmic staining. Untreated MDA-MB-468 cells exhibit a normal morphology, with diffuse cytoplasmic TMRE staining indicating a functional polarised mitochondrial membrane and have uncondensed chromatin, as evidence by uniform hoechst 33342 nuclear staining (Figure 5.5A).

Treating the MDA-MB-468 cell line with 1µM ANS for 24 hours, induced significant loss of TMRE staining with chromatin condensation evident by the densely packed chromatin within apoptotic bodies (Figure 5.5B). MDA-MB-468 cells that were treated for 48 hours with 1µM ANS or for 4 hours with 50µM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone CCCP, a known inducer of mitochondrial membrane depolarisation and loss of mitochondrial membrane potential was measured by staining cells in TMRE. Loss of mitochondrial membrane potential was measured by flow cytometry. A representative image of one experiment (n=3 in total), showing the increase in cells with ANS and CCCP induced mitochondrial membrane depolarisation can be seen in (Figure 5.5C). At a concentration of 1µM, ANS induced a 50% increase in the number of cells with depolarised mitochondria, 50µM CCCP induced an approximate 75% increase in the number of cells with depolarised mitochondria (Figure 5.5D). Thus, ANS can induce both chromatin condensation and mitochondrial membrane depolarisation, two hallmarks associated with apoptotic cell death.



5.5 ANS induces chromatin condensation and depolarisation of the mitochondrial membrane - A) MDA-MB-468 cells show normal chromatin staining indicated by the Hoechst 33342 (blue) and mitochondrial membrane potential as indicated by diffuse TMRE staining (red). **B)** MDA-MB-468 cells treated with 1µM ANS for 24 hours exhibit chromatin condensation as indicated by dense hoechst 33342 staining. **C)** Data representative of one experiment illustrating that 1µM ANS induced mitochondrial membrane depolarisation. **D)** MDA-MB-468 cells treated for 24 hours with 1µM ANS induces a 50% increase in the number of cells with mitochondrial membrane depolarisation. The positive control CCCP induced an approximate 70% increase in the number of cells with depolarised mitochondrial membranes. (Data shown comparing drug treatments to untreated control, n=3 ± SEM, one way ANOVA followed by Tukeys post hoc test. *P<0.05, **P<0.01).

The ability of the caspase inhibitor QVD-OPh to inhibit ANS induced chromatin condensation was tested in the MDA-MB-468 cell line. Cells were pre-treated with 40µM QVD-OPh for 1 hour, after this time, cells were co-treated and incubated for 24 hours in the presence of 40µM QVD-OPh and 1µM ANS. Following the incubation step, cells were fluorescently stained with the nuclear dye Hoechst 33342 and TMRE for 15 minutes and visualised using a fluorescent microscope. ANS induced chromatin condensation and this effect was inhibited by the caspase inhibitor QVD-OPh (Figure 5.6), suggesting a role of caspases in ANS induced chromatin condensation.

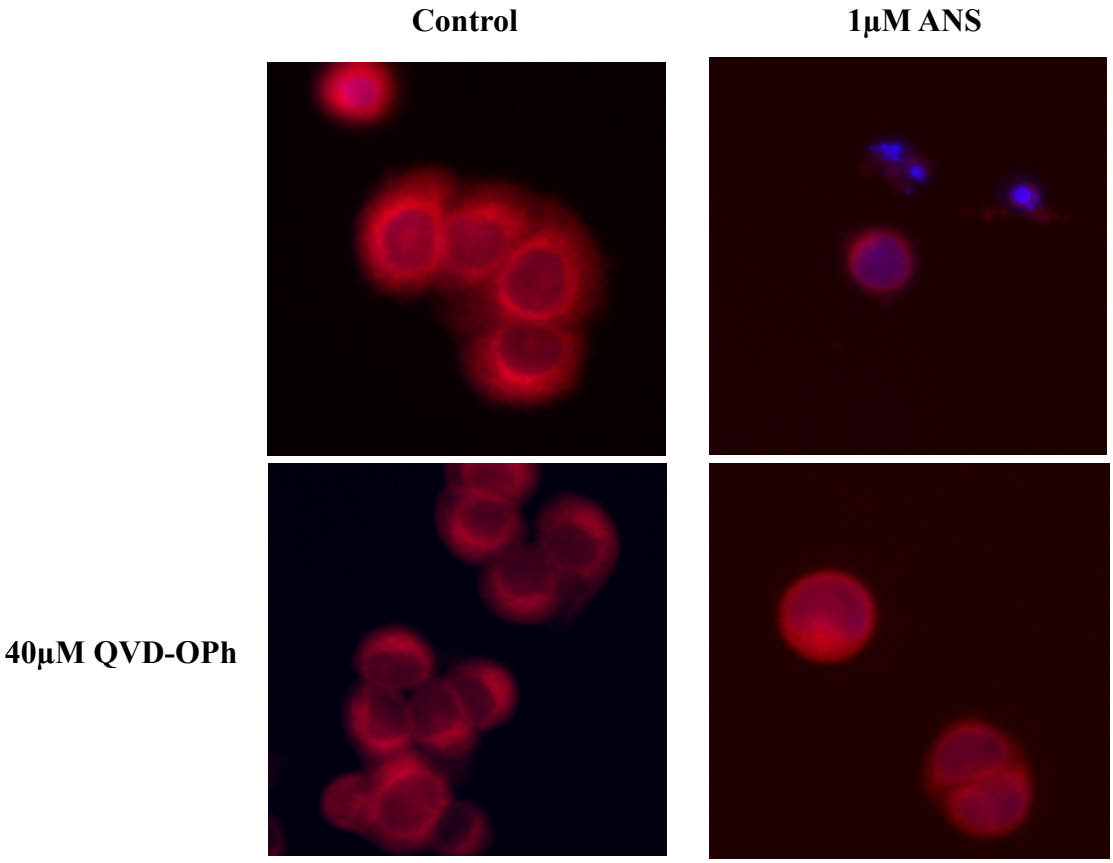


Figure 5.6 - ANS-induced chromatin condensation can be blocked with QVD-OPh - MDA-MB-468 cells were treated with either 1µM ANS, 40µM QVD-OPh or a combination of 1µM ANS and 40µM QVD-OPH, cells were incubated for 24 hours. Cells were then treated with TMRE and Hoechst 33342 and fluorescent microscope images at 20x magnification were taken. Untreated MDA-MB-468 cells exhibit normal TMRE and Hoechst 33342 staining. ANS treated cells displayed chromatin condensation as evidenced by intense Hoechst 33342 staining with concurrent loss of TMRE staining. The caspase inhibitor QVD-OPh inhibited ANS induced chromatin condensation as evidenced by the lack of dense hoechst 33342 staining.

5.2.4 *ANS induces low levels of caspase-3 processing and caspase-3 like activity*

Apoptosis is the main mode of cell death induced by ANS in cancer cell lines *in vitro,* that is reported in the literature (Curtin and Cotter, 2002, Ruller et al., 1999, Mawji et al., 2007). Thus, the ability of ANS to induce caspase-3 processing and caspase-3 like activity in the TNBC cell line MDA-MB-468 was tested. The MDA-MB-468 cell line was treated with 1μM ANS or 10μM VP16 (etoposide) a known inducer of caspase processing and activation and cells were incubated in the presence of each drug for 24 or 48 hours. At each time-point samples were harvested for the detection of caspase processing by immunoblotting. Treatment of the MDA-MB-468 cell lines with 1μM ANS induced very low levels of caspase-3 processing at both the 24 and 48 hour time-points, with only small levels of the 17kDa fragment being generated (Figure 5.7A), indicative of low levels of caspase-3 activity. VP16 exhibited low levels of caspase-3 processing at the 24 hour time-point, however, at the 48 hour time-point VP16 induced significantly greater levels of caspase-3 processing and generation of the 17kDa cleavage fragment (Figure 5.7A) indicative of high levels of caspase-3 activity. The ability of ANS treatment to induce caspase-3 like activity was tested to validate the findings from the immunoblot. The MDA-MB-468 cell line was treated with 3μM ANS for 24 hours. Treatment of the MDA-MB-468 cell line in 3μM ANS induced a four fold increase in fluorescence activity indicating low levels of active caspase-3 (Figure 5.7B). This low level of caspase-3 like activity coincides with the low levels of caspase-3 processing observed in response to ANS treatment.

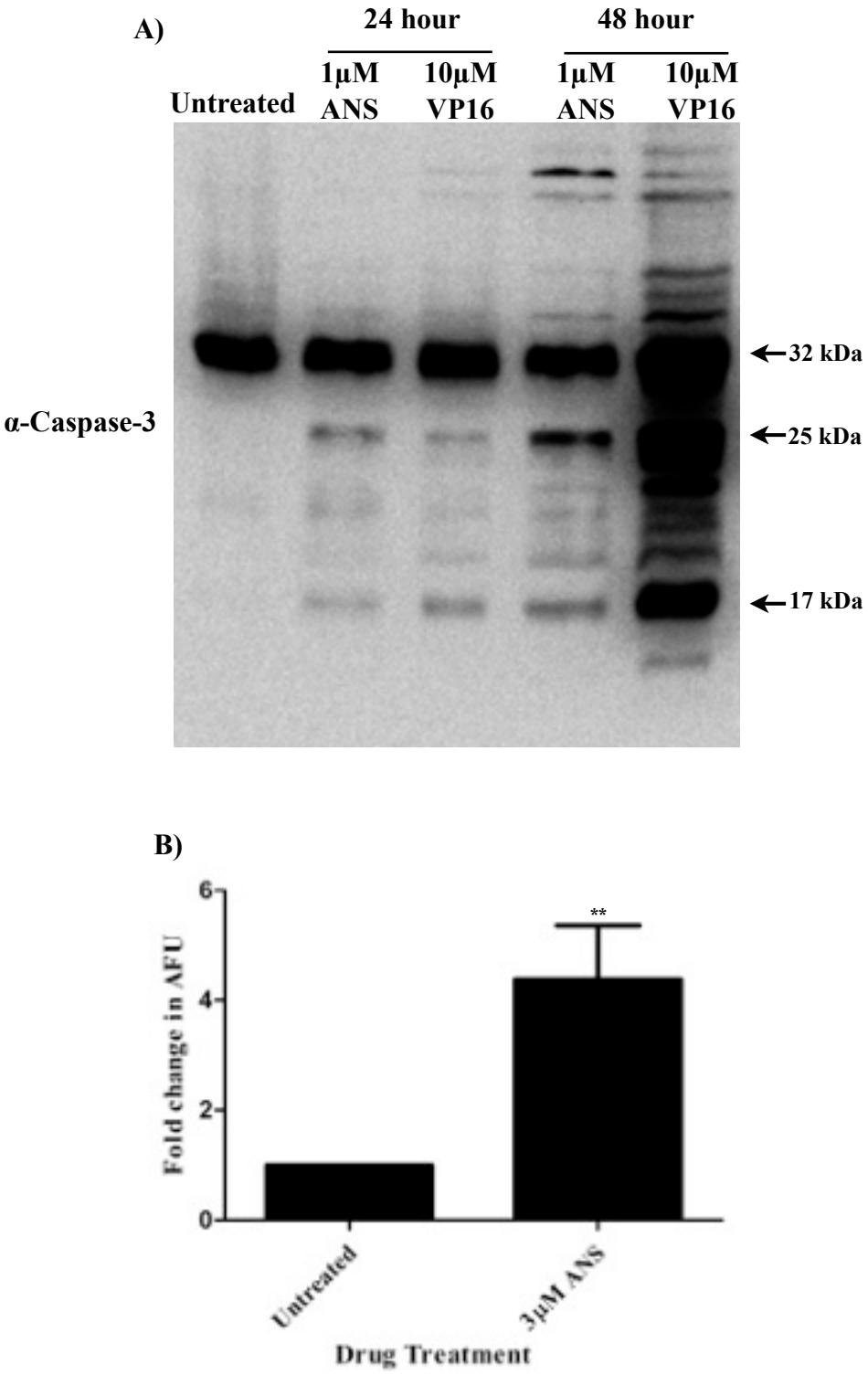


Figure 5.7 - ANS induces caspase 3 processing and caspase-3 like activity in MDA-MB-468 cells - The MDA-MB-468 cell line were treated with 1μM ANS or 10μM VP16. Cells were incubated in the presence of the drugs for either 24 or 48 hours, after each time-point cells were harvested and prepared for western blotting. **A)** Both 24 and 48 hour time-points of 1μM ANS treatment induced low levels of caspase-3 processing and generation of the catalytically active fragments. VP16 (etoposide) induces significantly higher levels of caspase-3 processing and generation of cleavage fragments, indicative of caspase activation. **B)** The MDA-MB-468 cell line was treated with 3μM ANS for 24 hours after this time cells were harvested and a caspase-3 like activity assay was performed. 3μM ANS induced a four fold increase in caspase-3 like activity in the MDA-MB-468 cell line. (Data shown comparing ANS treatment to untreated control. n=3 ± SEM, one way ANOVA followed by Tukeys post hoc test. **P<0.01).

The ability of QVD-OPh to protect MDA-MB-468 cells against ANS-induced decrease in cellular reductase activity and cell number was tested. MDA-MB-468 cells were pretreated with 10μM QVD-OPh for one hour and subsequently co-treated with 1μM ANS and 10μM QVD-OPh for either 24 or 48 hours. At each time-point, cell number was measured and the alamar blue assay was performed at the 48 hour time-point only. Co-treatment of the MDA-MB-468 cell line for 48 hours with 10μM QVD-OPh, failed to protect cell from ANS-mediated decrease in cellular reductase activity, as co-treated cells exhibited the same decrease in cellular reductase activity as 1μM ANS alone (Figure 5.8A). Treatment of the MDA-MB-468 cell line with 1μM ANS resulted in a statistically significant two fold decrease at the 24 hour time-point and four fold decrease at the 48 hour time-point in cell number (Figure 5.8B). Pre-treatment of the MDA-MB-468 cell line with 10μM QVD-OPh and subsequent co-treatment of 1μM ANS and 10μM QVD-OPh did not rescue cells from the ANS-induced decrease in cell number at either the 24 or 48 hour time-point (Figure 5.8B). In addition, the MDA-MB-468 cell line was pre-treated with 10μM QVD-OPh and subsequently co-treated and incubated with 3μM ANS and 10μM QVD-OPh for 24 hours. After this time cells were washed with medium, fresh medium was added and cells were incubated in medium for seven days. Following the seven day incubation, a colony formation assay was performed and the number of colonies counted. Treating the MDA-MB-468 cell line with 3μM ANS resulted in an approximate 20 fold reduction in the number of colonies compared to untreated MDA-MB-468 cells. Co-treating MDA-MB-468 cells with 3μM ANS and 10μM QVD-OPh (caspase inhibitor), did not protect the ANS-induced 20 fold reduction in colony number (Figure 5.8C). To ensure that 10μM QVD-OPh is a sufficient concentration to block caspase-3 like activity, the ability of 10μM QVD-OPh to inhibit VP16-induced caspase-3 like activity was tested. The MDA-MB-468 cell line was pre-treated with 10μM QVD-OPh for one hour and subsequently cells were co-treated with 10μM QVD-OPh and 10μM VP16. 10μM VP16 induced an approximate four fold increase in caspase-3 like activity which was completely blocked in the presence of 10μM QVD-OPh (Figure 5.8D). Thus, illustrating that 10μM QVD-OPh is sufficient to inhibit caspase-3 like activity.

These data suggest that caspases can be activated in response to ANS treatment and induction of apoptosis may contribute to ANS mediated cell death, however, blocking apoptosis does not block cell death. To determine the morphological changes that occur in response to ANS treatment, transmission electron microscopy (TEM) images of MDA-MB-468 cells treated with ANS and VP16 in the absence and presence of QVD-OPh were taken.

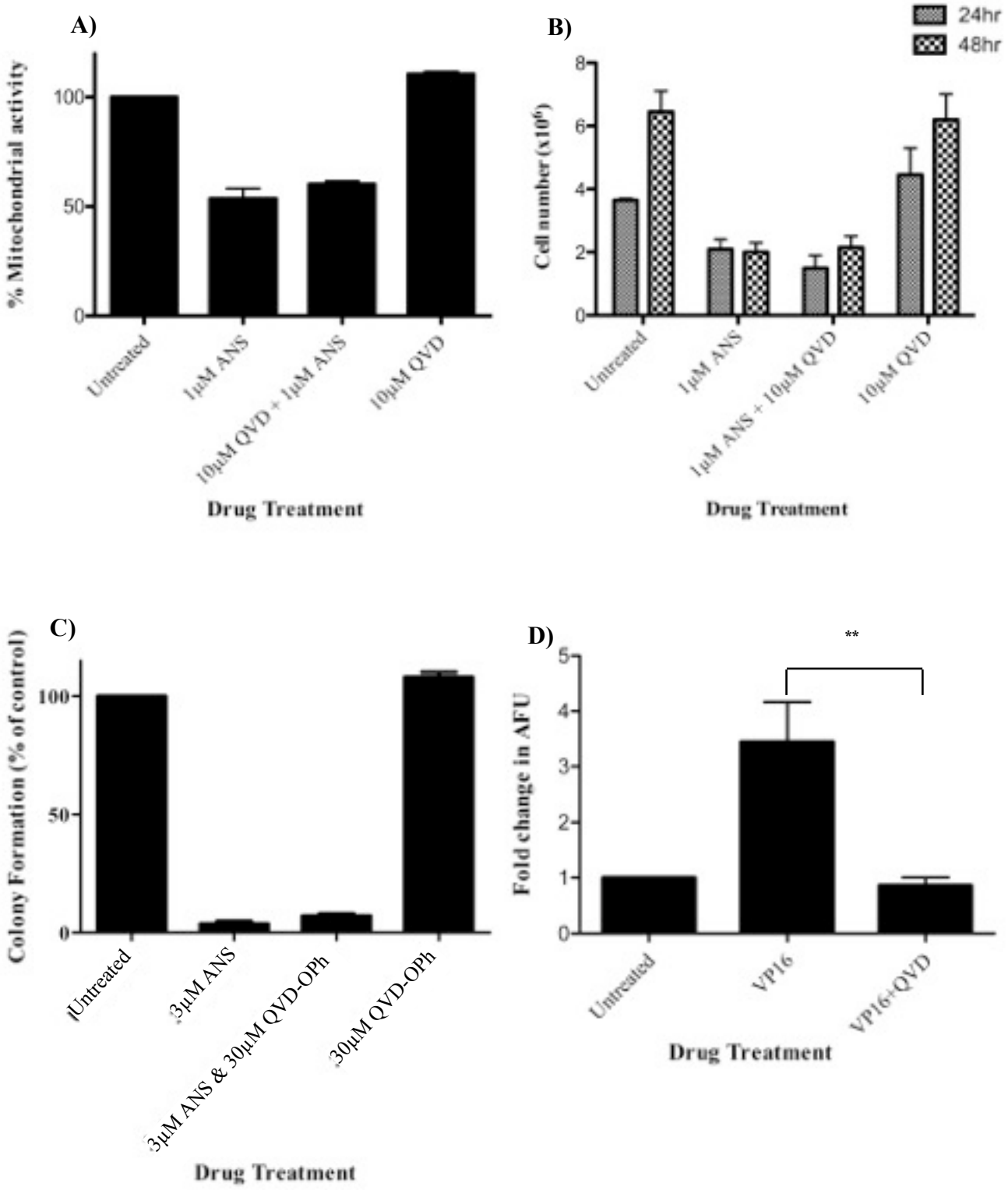
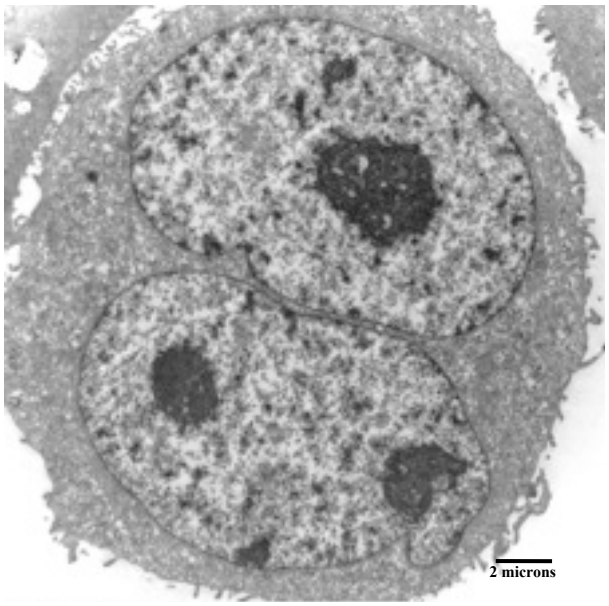


Figure 5.8 - The pan caspase inhibitor QVD-OPh does not protect MDA-MB-468 cells from ANS induced decrease in cellular reductase activity, cell number or colony formation - MDA-MB-468 cells were treated with ANS with or without 10μM QVD-OPh or with QVD-OPh alone. Cells were incubated for 48 hours in the presence of the drug and subjected to the alamar blue assay. MDA-MB-468 cells were also treated as above and harvested after 24 and 48 hours. At each time-point the cell number was measured. **A)** 1μM ANS induced a statistically significant decrease in cellular reductase activity ($P<0.001$), this ANS mediated decrease was not inhibited by pre-incubating cells with the pan caspase inhibitor QVD-OPh. **B)** 1μM ANS also induced a statistically significant decrease in cell number ($P<0.001$). This ANS mediated decrease in cell number was not rescued by pre-incubating MDA-MB-468 cell line in 10μM QVD-OPh. **C)** The caspase inhibitor QVD-OPh did not protect MDA-MB-468 cells from ANS induced 20 fold decrease in colony formation. **D)** 10μM QVD-OPh is sufficient to decrease the approximate four fold increase in VP16 induced caspase-3 like activity. (Data shown n=3 ± SEM, two way ANOVA, followed by Bonferroni post hoc test. **D)** one way ANOVA followed by Tukeys post hoc test **$P<0.01$).

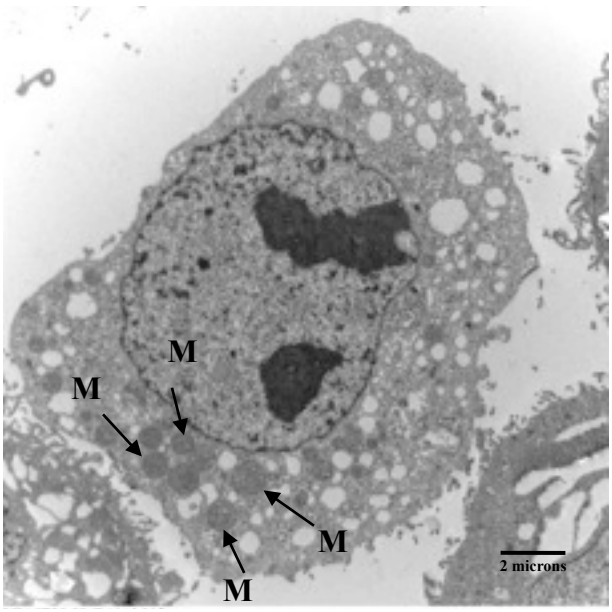
5.2.5 ANS treated MDA-MB-468 cells display apoptotic and autophagic-like morphologies

To identify the mode(s) of cell death induced in the MDA-MB-468 cell line, cells were treated with 1μM ANS or 30μM VP16 for 24 and 48 hours. After this time, cells were prepared for TEM to determine any morphological changes that occur and identify if the morphology is consistent with a particular form of cell death. Untreated MDA-MB-468 cells exhibited normal nuclear and cytoplasmic morphology (Figure 5.9A). Treating the MDA-MB-468 cell line with 30μM VP16, a well characterised activator of caspase-3 and apoptosis, induced chromatin condensation, abnormal mitochondria as evidenced by their electron dense appearance and cytoplasmic vacuolisation after 24 hour incubation (Figure 5.9B). Late stage apoptosis is evident with compartmentalisation of intracellular components, membrane blebbing, cell shrinkage and clear cellular dismantling after 48 hour incubation in 30μM VP16 (Figure 5.9C). 1μM ANS induced chromatin condensation, compartmentalisation of intracellular components and membrane blebbing after 24 hour treatment, morphologies similar to VP16 and are consistent with apoptosis (Figure 5.9D). However, the majority of 1μM ANS treated MDA-MB-468 cells displayed a morphology that was distinct from apoptosis. A large proportion of ANS treated cells were found to have incomplete chromatin condensation, cytoplasmic vacuolisation (Figure 5.9E) and aggregation of lysosomes (Figure 5.9F). Additionally, ANS-induced vacuoles appear to contain intracellular components (Figure 5.9G). Figure 5.9H, is a representative image from a field of view containing ANS treated cells with both apoptotic and non-apoptotic morphologies. Interestingly, there were a number of examples of cells that had undergone apoptotic cell death and had been phagocytosed by a “normal” neighbour cell (Figure 5.9I). These morphological changes occurring in the MDA-MB-468 cell line in response to ANS treatment are consistent with cells undergoing apoptosis and cells that display an autophagy-like morphology.

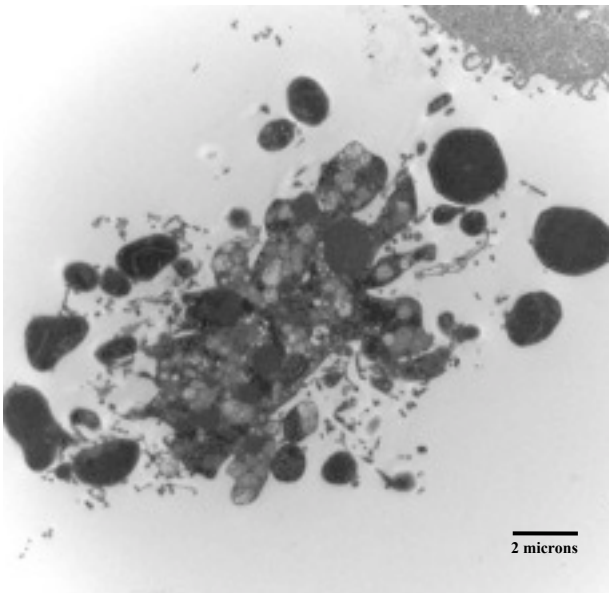
A) Untreated



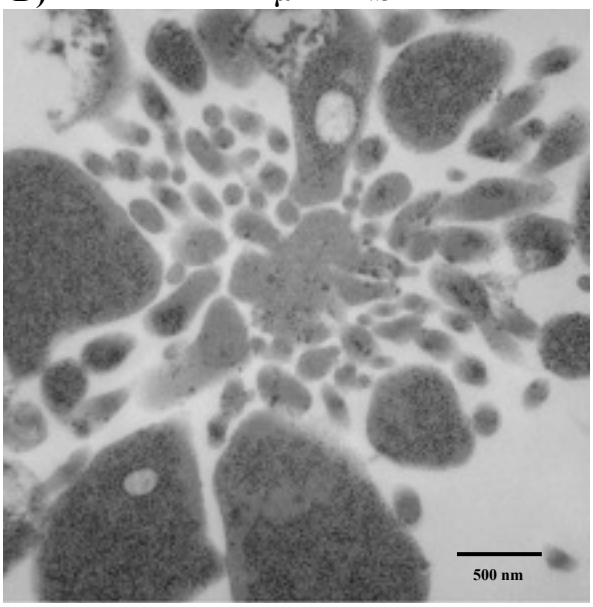
B) 24hr 30µM VP16



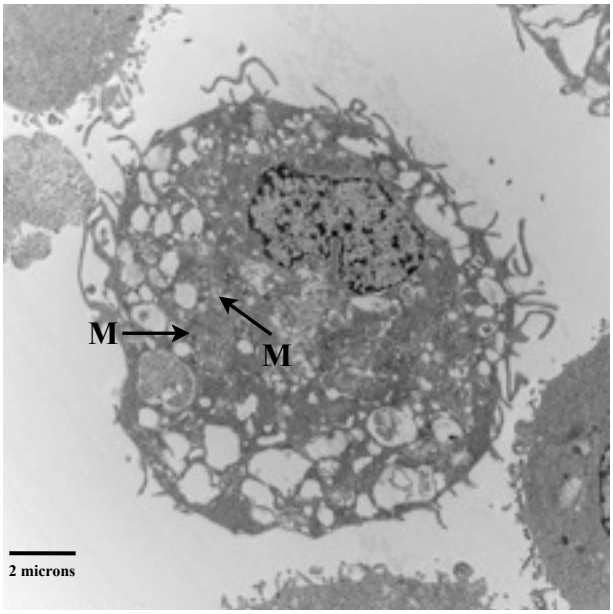
C) 48hr 30µM VP16



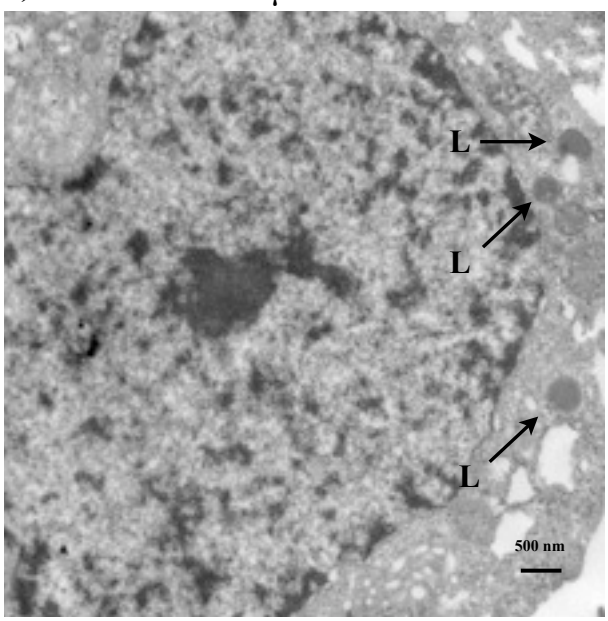
D) 24hr 1µM ANS



E) 24hr 1µM ANS



F) 24hr 1µM ANS



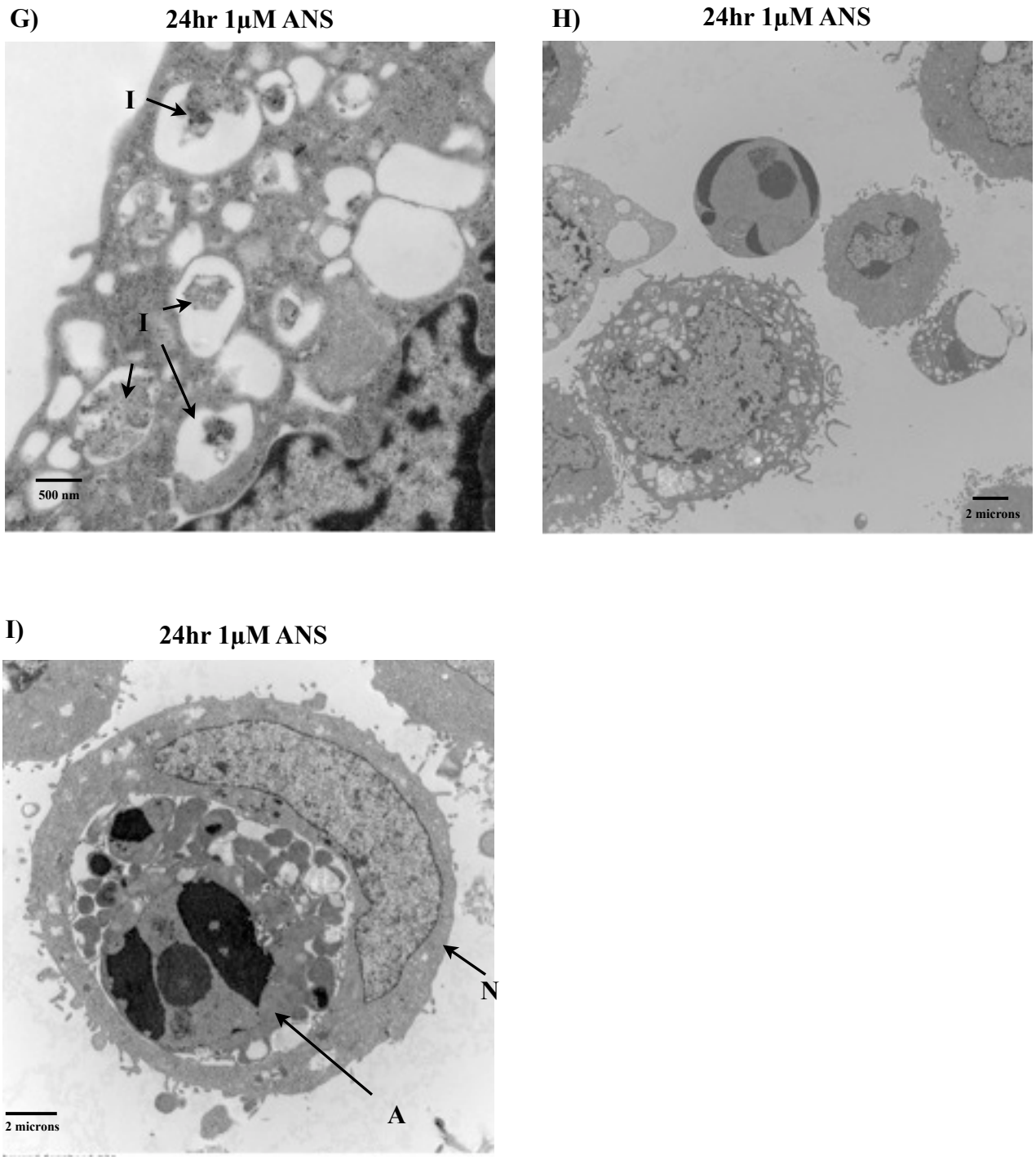


Figure 5.9 - MDA-MB-468 cells undergoing ANS induced death display a morphology consistent with apoptosis and a morphology that is autophagy-like - MDA-MB-468 cells were treated for 24 hours with ANS or 24 and 48 hours with VP16 as a positive control for apoptosis. **A)** Untreated MDA-MB-468 cells have normal nuclear and cytoplasmic morphologies. **B)** 24 hour VP16 treatment induces vacuolisation of the cytoplasm with abnormal mitochondria as evidenced by their visible electron density (indicated by black arrows). **C)** 48 hour VP16 treatment induces formation of apoptotic bodies and is evidence of late stage apoptosis. **D)** 24 hour ANS induced formation of apoptotic bodies illustrating the presence of late stage apoptotic cells. **E)** ANS induces significant cytoplasmic vacuolisation and incomplete chromatin condensation. **F)** ANS treated cell exhibits vacuoles and aggregation of lysosome-like structures (indicated by arrows). **G)** High magnification of cytoplasmic vacuoles which appear to contain intracellular components. **H)** Representative field of view image illustrating distinct morphologies. **I)** Apoptotic cell that has been phagocytosed by a neighbouring cell. (L=lysosomal like structure, M=mitochondria, I=cytoplasmic inclusion, A=apoptotic cell and N=normal cell).

The next step was to determine if the caspase inhibitor QVD-OPh, could prevent induction of VP16 and ANS induced morphological changes in the MDA-MB-468 cells. Untreated MDA-MB-468 cells displayed a normal cellular morphology (Figure 5.10A). Treatment with 10μM VP16 induced classical apoptotic morphology with chromatin condensation, cell shrinkage and membrane blebbing (Figure 5.10B). The appearance of this apoptotic morphology was inhibited following addition of 10μM QVD-OPh and these cells display a normal cellular morphology (Figure 5.10C). Treating cells with 1μM ANS, induced incomplete chromatin condensation and cytoplasmic vacuolisation (Figure 5.10D). Addition of 10μM QVD-OPh to ANS treated cells reversed incomplete chromatin condensation and resulted in the formation of smaller cytoplasmic vacuoles. Interestingly, co treatment of 1μM ANS and 10μM QVD-OPh appears to induce increased aggregation of lysosomal like structures (Figure 5.10E).

Thus, it appears that ANS induces apoptosis in some MDA-MB-468 cells as evidenced by morphologies consistent with apoptosis and shares similarities with VP16 treated MDA-MB-468 cells. The ability of ANS to induce apoptosis correlates with the low levels of caspase-3 like activity, caspase-3 processing and chromatin condensation that could be blocked by 10μM QVD-OPh illustrated earlier. However, ANS treatment also induced a distinct cellular morphology which exhibits cytoplasmic vacuolisation, appearance of lysosomal like structures and incomplete chromatin condensation. This cellular morphology shares a number of similarities that are consistent with autophagy. Whether cells exhibiting this autophagy-like morphology are undergoing cell death is not deducible from this experiment. However, taking the previous data into consideration, it does appear that cells are undergoing caspase-independent cell death. ANS can induce caspase-dependent apoptosis which is blocked by QVD-OPh. In addition, ANS can induce caspase-independent cell death and these cells are morphologically dissimilar to apoptotic cells, but instead display morphologies similar to autophagy.

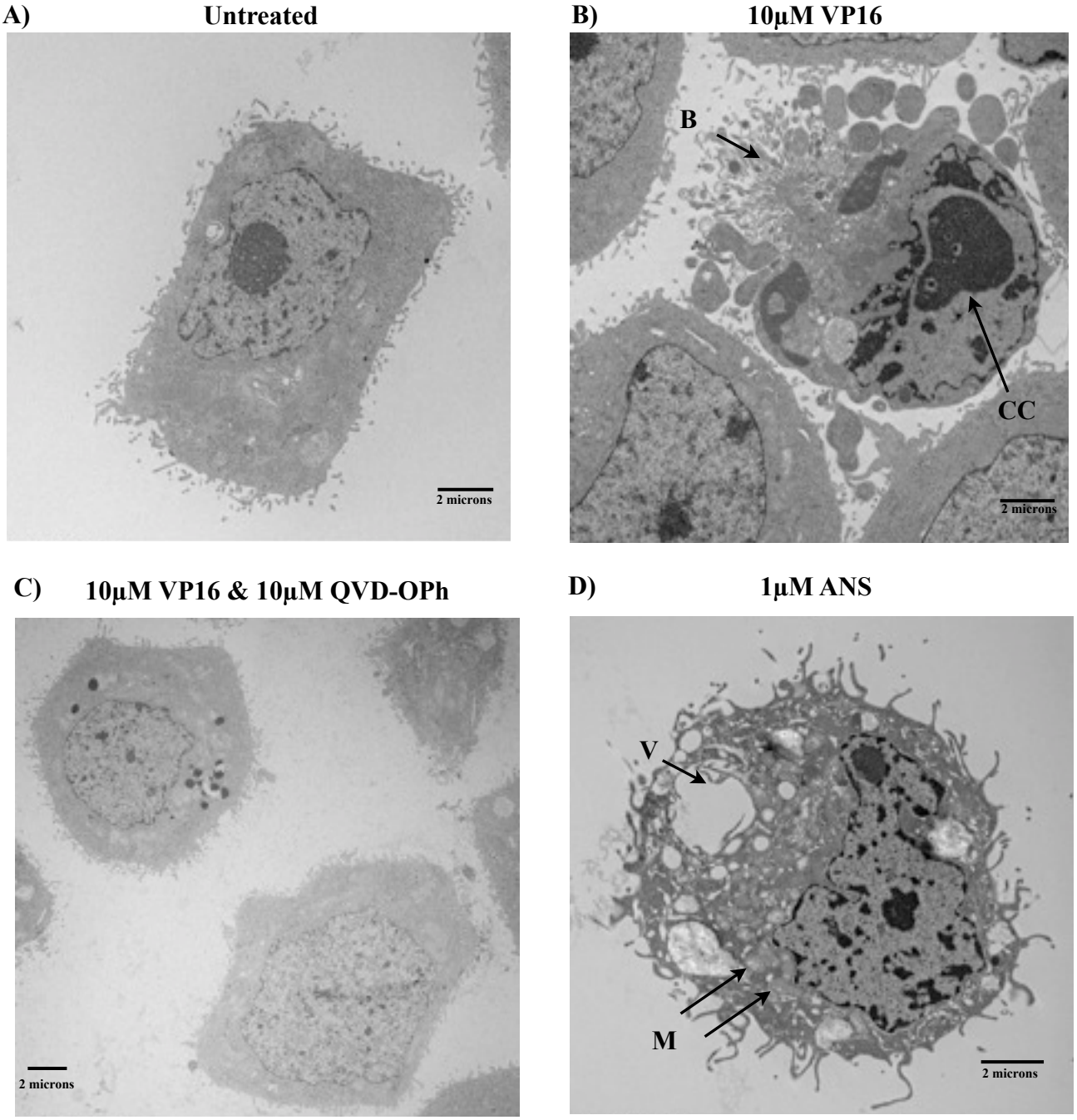


Figure 5.10 - The caspase inhibitor QVD-OPh does not protect MDA-MB-468 cells from ANS-induced cytoplasmic vacuolisation - MDA-MB-468 cells were treated for 24 hours with ANS or VP16 and co-treated with and without the caspase inhibitor QVD-OPh **A)** Untreated MDA-MB-468 cells have normal nuclear and cytoplasmic morphologies, **B)** VP16 treatment induces cytoplasmic blebbing and chromatin condensation, **C)** Co-treating QVD-OPh with VP16 protected MDA-MB-468 cells from undergoing apoptosis and cells display a normal cellular morphology, **D)** ANS induces significant vacuolisation which appear to contain intracellular debris and chromatin appears condensed, **E)** QVD-OPh and ANS co-treated cells appear larger that ANS alone treatment, cells have normal nuclear morphology but cytoplasmic vacuolisation persists with aggregation of lysosomal like structures. (B=blebbing, CC=condensed chromatin, V=vacuole, M=mitochondria and L=lysosomal like structure)

5.2.6 *ANS decreases MCL-1 levels in both ANS sensitive MDA-MB-468 and insensitive Hs578t TNBC cell lines but is not responsible for ANS-induced decrease in cellular reductase activity*

ANS binds to the 60S subunit of functional 80S ribosomes, inhibiting peptidyl transferase activity and subsequently shutting down protein synthesis in cells. The concentration of ANS and another protein synthesis inhibitor CHX to affect protein synthesis inhibition was determined by measuring the short half life anti-apoptotic protein MCL-1. MDA-MB-468 cells were treated with either ANS or CHX for four hour, after this time cells were harvested and immunoblotting for MCL-1 was performed. ANS decreased the expression of MCL-1 protein at a concentration of 100nM (Figure 5.11A). CHX also decreased MCL-1 protein expression at a concentration of 1µM (Figure 5.11B). Thus, both ANS and CHX are capable of decreasing MCL-1 protein expression and correlates with the ability of ANS and CHX to inhibit protein synthesis as reported in the literature.

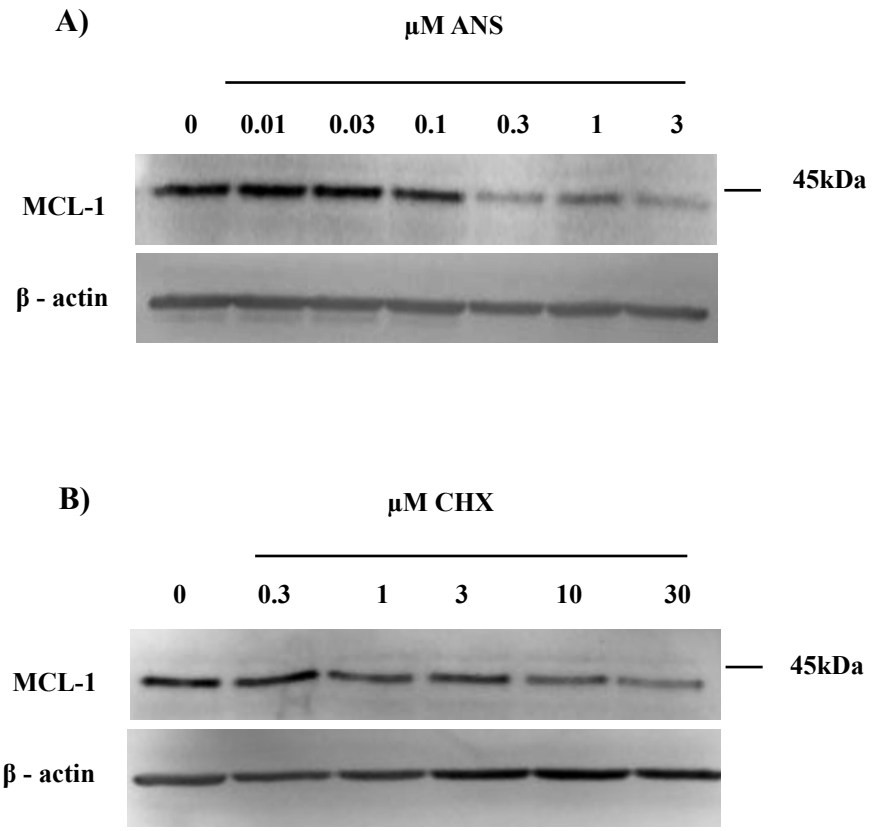


Figure 5.11 - The protein synthesis inhibitors ANS and CHX decrease the expression levels of the short half-life anti-apoptotic protein MCL-1 - A) The MDA-MB-468 cell line was treated for four hours with increasing concentrations of ANS. Cells were harvested and immunoblotted for MCL-1 protein. ANS decrease MCL-1 protein at concentrations greater than 0.3µM. **B)** The MDA-MB-468 cell line was treated for four hours with increasing concentrations of CHX. Cells were harvested and immunoblotted for MCL-1 protein. CHX decreased MCL-1 protein expression at concentrations greater that 3µM. (Data shown representative of n=3).

Next, the ability of ANS to differentially effect the expression of MCL-1 was tested in the ANS sensitive MDA-MB-468 and ANS insensitive Hs578t TNBC cell lines. MDA-MB-468 and Hs578t TNBC cell lines were incubated with either 1μM ANS or 10μM CHX for four hours. After this time cells were harvested, protein concentration determined and immunoblotting was performed for MCL-1. Treatment of both cell lines with 1μM ANS induced a significant decrease in the levels of MCL-1. Treating cells with 10μM CHX, also induced a significant decrease in MCL-1 expression levels in both cell lines (Figure 5.12A). Both the MDA-MB-468 and Hs578t cell lines were treated with 1μM ANS and 10μM CHX and incubated in each drug for 48 hours, after which time the alamar blue assay was performed. MDA-MB-468 cells were more sensitive than the Hs578t cell lines to both ANS and CHX (Figure 5.12B). ANS is more effective than CHX at decreasing cellular reductase activity in both the MDA-MB-468 and Hs578t cell lines, however the sensitivities of each cell line to ANS and CHX was comparable. The colorectal cancer cell line HT-29, was shown to be insensitive to ANS at concentrations up to 100μM as determined by the alamar blue assay (Figure 5.2B). Thus, the ability of ANS and CHX to affect MCL-1 protein expression and colony formation was tested. HT-29 cells were seeded and incubated for four hours in 3μM ANS or 30μM CHX, after this time cells were harvested and immunoblotting for MCL-1 protein was performed. Both ANS and CHX significantly decrease MCL-1 protein expression in the HT-29 cell line, thus correlating with inhibition of protein synthesis (Figure 5.12C). In parallel, HT-29 cells were incubated for 24 hours in different concentrations of ANS or CHX, after this time the drugs were washed off, fresh medium was added and cells were incubated for a further seven days. Following this incubation step a colony formation assay was performed. Neither ANS nor CHX at concentrations of 30μM induced a statistically significant decrease in HT-29 colony number (Figure 5.12C). Thus, despite both ANS and CHX decreasing MCL-1 protein expression in the HT-29 cell line, there was no change in colony formation. In summary, while this data on its own, does not illustrate definitively that protein synthesis is inhibited, this data does correlate with published literature and suggests that ANS and CHX inhibit protein synthesis in the MDA-MB-468 Hs578t and HT-29 cell lines. However, inhibition of protein synthesis does not correlate with decreased cellular reductase activity or colony formation.

MCL-1 is known to be over-expressed in certain cancers and over-expression correlates with development of drug resistance (Krajewska et al., 1996a, Krajewska et al., 1996b, Zhou et al., 2001). CHX decreased MCL-1 to a similar level as ANS however, CHX was less effective than ANS at decreasing cellular reductase activity. To further rule out MCL-1 playing a role in the decreased cellular reductase activity induced by ANS, MCL-1 expression was knocked down using siRNA in the MDA-MB-468 cell line. MDA-MB-468 cells were transfected with 25nM MCL-1 siRNA and cells were incubated in the presence of the siRNA for 48 hours, after this time the alamar blue assay was performed. siRNA mediated knockdown of MCL-1 expression did not induce a statistically significant decrease in cellular reductase activity, despite a significant reduction in MCL-1 protein expression (Figure 5.12D). This illustrates that the ANS induced decreased of MCL-1 protein expression, does not contribute to the decrease in cellular reductase activity and cell death induced by ANS.

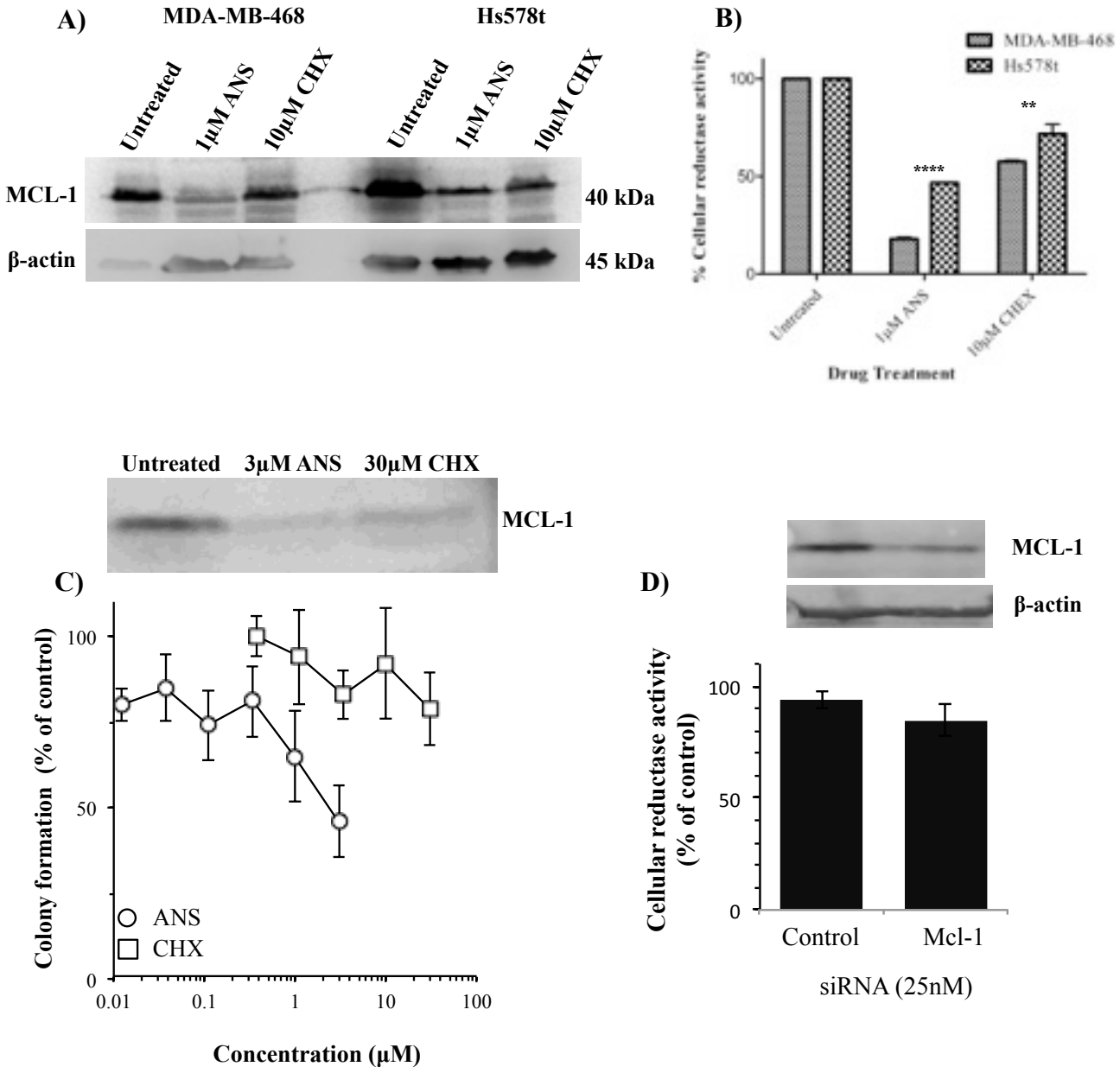


Figure 5.12 - ANS and CHX decrease MCL-1 expression in sensitive MDA-MB-468 and insensitive Hs578t and HT-29 cell lines - A) The MDA-MB-468 and Hs578t TNBC cell lines were treated with either 1μM ANS or 10μM CHX for four hours, after this time cells were harvested and western blotting performed. Both ANS and CHX significantly decreased the expression of MCL-1 in both the ANS sensitive and ANS insensitive TNBC cell lines. There is slight variation in the levels of β-actin between the cell lines however, the decrease in MCL-1 levels is evident and is not due to differences in protein loading. **B)** The MDA-MB-468 and Hs578t cell lines were treated with either 1μM ANS or 10μM CHX and incubated in the presence of each drug for 48 hours and the alamar blue assay was performed. 1μM ANS induced a statistically significant decrease in cellular reductase activity of both the MDA-MB-468 and Hs578t with the MDA-MB-468 cell line being more sensitive to ANS treatment. The protein synthesis inhibitor CHX induces a statistically significant decrease in cellular reductase activity in both the MDA-MB-468 and Hs578t cell lines, with the MDA-MB-468 cell line being more sensitive **C)** Four hour incubation of the colorectal cancer cell line HT-29, in either 3μM ANS or 30μM CHX, significantly decreased expression of MCL-1 protein. A concentration response of ANS and CHX was performed using the HT-29 cells, which were incubated in each drug for 24 hours. After this time drug was washed off, fresh medium was added and cell were incubated for a further seven days. Following this incubation period, a colony formation assay was performed. At concentrations of 3μM ANS or 30μM CHX, failed to induce a statistically significant decrease in colony number in the HT-29 cell line. (equal protein loading was determined by bradford and confirmed using Ponceau S). **D)** The MDA-MB-468 cell line was treated with 25nM MCL-1 siRNA for 48 hours. After this time the alamar blue assay was performed and MCL-1 protein levels were determine. MCL-1 siRNA failed to induce a statistically significant decrease in cellular reductase activity despite a significant reduction in the protein expression of MCL-1. (Data shown comparing drug treatments between cell lines, n=3 ± SEM followed by two way ANOVA and bonferroni post hoc test. **P<0.01, ****P<0.0001).

5.2.7 ANS induces phosphorylation of JNK but not p38 in the ANS sensitive MDA-MB-468 and insensitive Hs578t TNBC cell lines

ANS is known to potently induce the phosphorylation and subsequent activation of the stress activated kinases JNK and p38 (Morton et al., 2003). Activation of the JNK and p38 protein kinases induces stress response signalling pathways, that are reportedly required for the induction of apoptosis in certain cancer cell lines (Ruller et al., 1999). Thus, the ability of ANS to induce phosphorylation of JNK and p38 in both ANS sensitive MDA-MB-468 and ANS insensitive Hs578t TNBC cell lines was tested. Both cell lines were treated with 1μM ANS for two, four and six hours, at each time-point cells were harvested for immunoblotting. ANS was found to strongly induce phosphorylation of the p54 and p46 JNK isoforms, in both the MDA-MB-468 and Hs578t cell lines after just two hours and elevated phosphorylation levels persisted for the duration of the six hour time-course (Figure 5.13A). The intensity of background-subtracted JNK phosphorylation in response to ANS treatment, normalised to total JNK levels, show that there is greater induction of phosphorylated JNK subunits p54 and p46 JNK in the MDA-MB-468 cell line, with the peak of JNK phosphorylation evident after two hours but persisting over the duration of the six hour time-course (5.13B and C). When background subtracted and normalised to total p38 levels, ANS was found not to induce phosphorylation of p38 in either the MDA-MB-468 and Hs578t cell lines (Figure 5.13A). Equal protein loading was confirmed using using β-actin (Figure 5.13A).

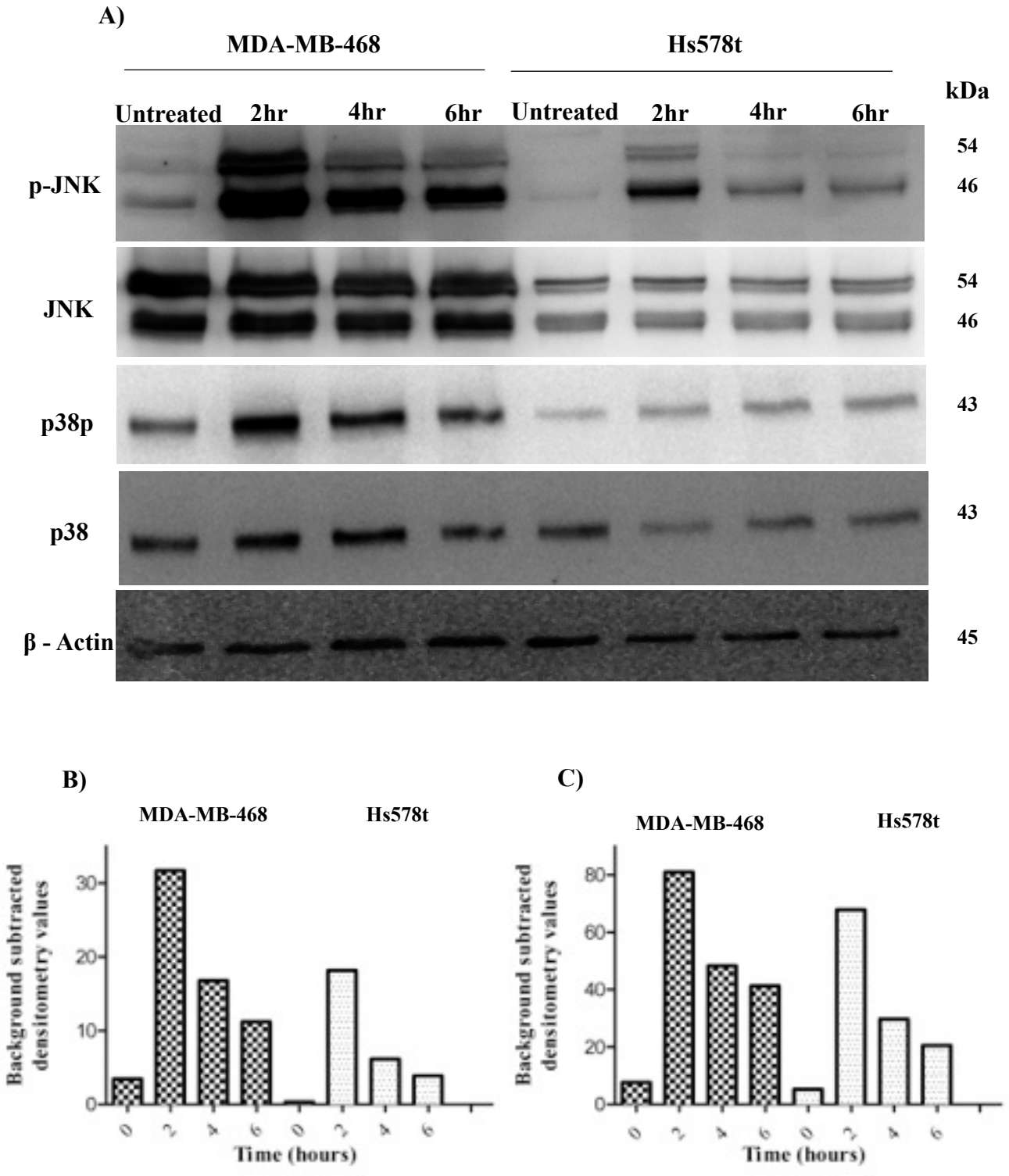
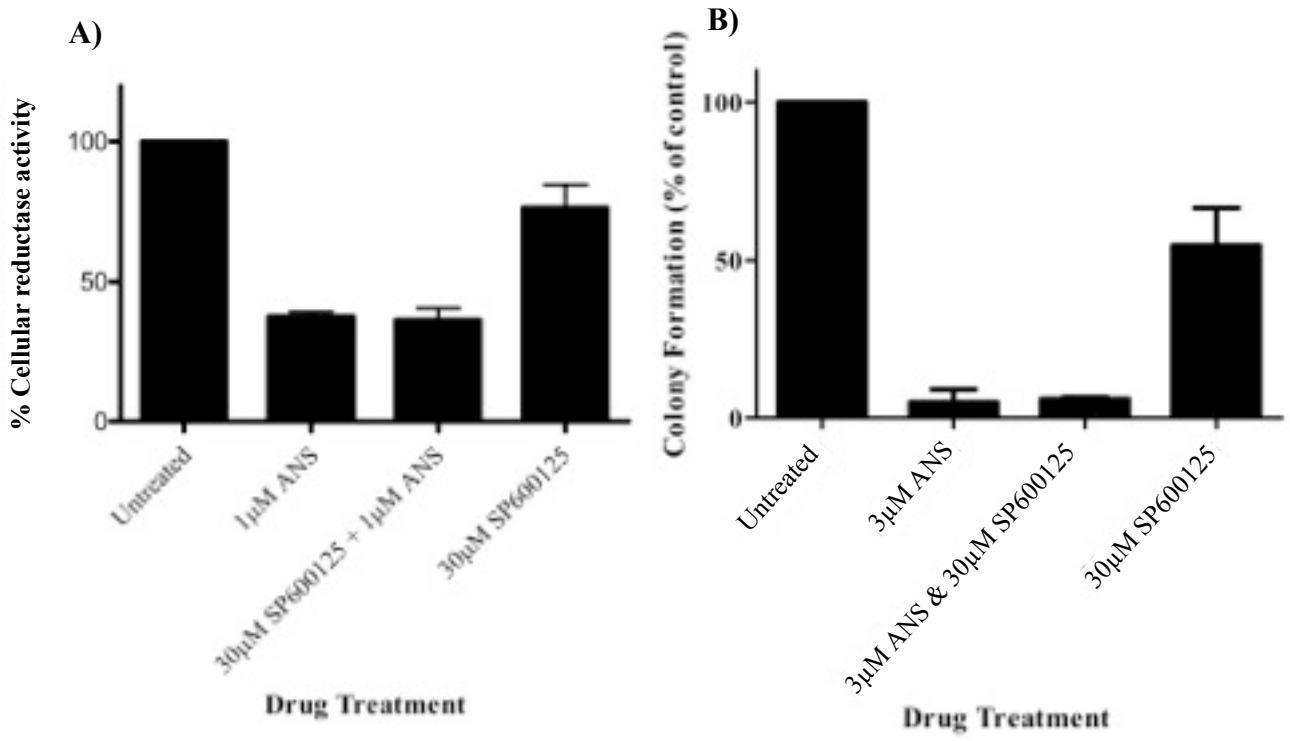


Figure 5.13 - ANS induces phosphorylation of JNK both in the ANS sensitive MDA-MB-468 and ANS insensitive Hs578t cell lines - A) ANS treatment induces significant phosphorylation of both JNK subunits p46 and p54 and also induces phosphorylation of p38 in MDA-MB-468 and Hs578t breast cancer cell lines. The intensity of p54 JNK subunit phosphorylation rapidly decays after two hours in both cell lines. Interestingly, phosphorylation of the p46 JNK subunit appears to persist in the ANS-sensitive TNBC cell line MDA-MB-468 compared to the ANS-insensitive TNBC cell line Hs578t. Despite equal protein loading it is evident that MDA-MB-468 cells possess significantly higher levels of both total JNK and total p38 compared to the insensitive Hs578t cells. β-actin levels illustrate equal loading. **B)** densitometry of p54 and **C)** p46 subunits of phosphorylated JNK. This data is background subtracted and normalised to total JNK. MDA-MB-468 cells have higher levels of JNK phosphorylation at all time-points compared to Hs578t cells. (Data shown n=1 representative of 3 similar experiments).

5.2.8 *A pharmacological inhibitor of JNK phosphorylation does not protect cells from ANS-induced decrease in cellular reductase activity*

To determine if JNK phosphorylation and subsequent activation is responsible for the decrease in cellular reductase activity induced by ANS, the MDA-MB-468 cell line was treated with a pharmacological inhibitor of JNK, SP600125. SP600125 has previously been reported to inhibit a number of the signalling mediators of the JNK signalling pathway and can protect JNK mediated apoptosis in hepatocyte cells (Schwabe et al., 2004). The MDA-MB-468 cell line was pre-treated for one hour in 30μM SP600125 and cells were subsequently co-treated with 30μM SP600125 and 1μM ANS for 48 hours. At this time-point, cells were harvested for immunoblotting and in parallel the alamar blue assay was performed. Treatment of the MDA-MB-468 cell line with ANS decreased cellular reductase activity by approximately 70% and co-treatment of 1μM ANS with 30μM SP600125 did not protect cells against the ANS-induced decrease in cellular reductase activity (Figure 5.14A). To determine if SP600125 could rescue ANS induced decrease in colony formation, the MDA-MB-468 cell line was co-treated with 3μM ANS and 30μM SP600125 and incubated in the presence of each drug for 24 hours. After this time, drug containing medium was removed, fresh medium added and cells were subsequently incubated for seven days. Following the seven day incubation the colony formation assay was performed. 3μM ANS induced an approximately 20 fold decrease in the number of MDA-MB-468 colonies formed compared to untreated control. Co-treatment of 30μM SP600125 with 3μM ANS did not protect against the loss of MDA-MB-468 colonies induced by ANS (Figure 5.14B). Co-treatment of the MDA-MB-468 cell line with 3μM ANS and 30μM SP600125 resulted in a significant decrease in ANS induced JNK phosphorylation (Figure 5.14C). Thus, despite significantly decreasing the levels of JNK phosphorylation, SP600125 was ineffective at preventing ANS-mediated decrease in cellular reductase activity or colony formation.



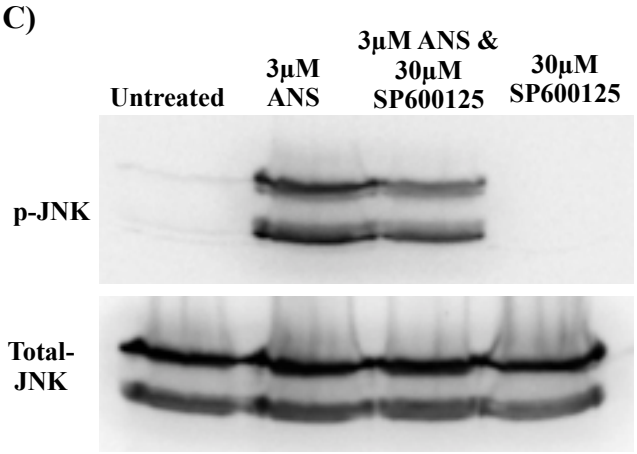


Figure 5.14 - The JNK inhibitor SP600125 does not prevent the ANS induced decrease in cellular reductase activity - A) Treatment of MDA-MB-468 cells with 1μM ANS induced a statistically significant decrease in cellular reductase activity ($P<0.001$). SP600125 did not prevent ANS mediated decrease in cellular reductase activity **B)** Treating MDA-MB-468 cells with 3μM ANS induced a statistically significant decrease in colony formation compared to untreated control ($P<0.01$). Co-treating 3μM ANS with 30μM SP600125 did not protect MDA-MB-468 cell from ANS inducing a statistically significant decrease in colony formation compared to ANS alone ($P<0.001$). **C)** ANS induces JNK phosphorylation and SP60125 significantly decreases ANS mediated JNK phosphorylation. (Data shown n=3 ± SEM, one way ANOVA followed by Tukeys post hoc test).

5.2.9 *The p38 inhibitor SB203580 does not protect cells from ANS induced decrease in cellular reductase activity*

The stress activated kinase p38 has been reported to be induced by ANS and can contribute to induction of apoptosis in some cell lines. SB203580 is a pharmacological inhibitor of p38 and is known to prevent phosphorylation and subsequent activation of p38 *in vitro* (Rolli et al., 1999). Despite ANS failing to induce detectable levels of p38 phosphorylation, the ability of the p38 inhibitor to protect the MDA-MB-468 cell lines from ANS induced decrease in cellular reductase activity was tested. MDA-MB-468 cells were pre-incubated for one hour with 10μM SB203580 and cells were subsequently co-treated with 1μM ANS and 10μM SB203580. Cells were incubated in the presence of each drug for 48 hours and after this time-point, cells were either harvested for immunoblotting or the alamar blue assay was performed. Treating the MDA-MB-468 cell line with 10μM SB203580 did not protect cells from ANS-mediated decrease in cellular reductase activity (Figure 5.15A). Immunoblotting for p38 phosphorylation in response to ANS treatment confirmed the result obtained in (Figure 5.10A) that p38 phosphorylation is not induced by ANS in the MDA-MB-468 cell line (Figure 5.15B). As determined by immunoblotting it appears that MDA-MB-468 cells have a high baseline of p38 phosphorylation under normal conditions.

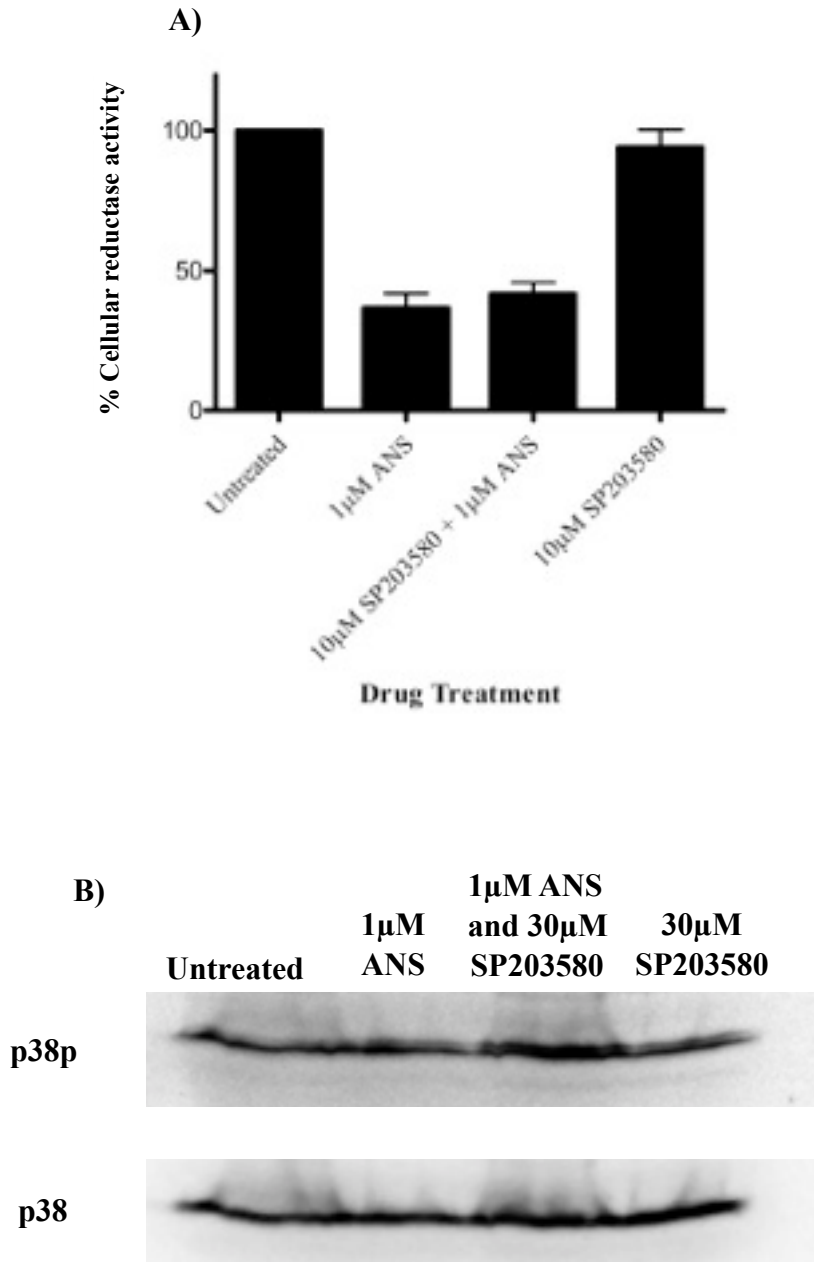


Figure 5.15 - The p38 inhibitor SP203580 does not prevent the ANS induced decrease in cellular reductase activity - A) The p38 inhibitor SP203580 did not protect the MDA-MB-468 cell line from ANS induced decrease in cellular reductase activity ($P<0.001$), **B)** The MDA-MB-468 cell line has high base levels of phospho-p38. Treating cells for 4 hours with 1μM ANS did not induce phosphorylation of p38 and co-treatment of the p38 inhibitor SP203580 failed to inhibit basal p38 phosphorylation. (Data shown n=3 ± SEM, one way ANOVA followed by Tukeys post hoc test).

5.2.10 *Testing the ability of ANS analogues, classified by their ability to induce JNK phosphorylation to decrease cellular reductase activity*

Following confirmation that ANS induced JNK phosphorylation, a collaboration with Dr. Alison Hulme was established in an attempt to determine the significance, if any, JNK phosphorylation contributes to the decreased cellular reductase activity mediated by ANS. Dr. Hulme kindly provided twelve ANS analogues classified based on their ability to induce phosphorylation and hence activation of the JNK kinase pathway (Figure 5.17). These ANS analogues were tested for their potential to decrease cellular reductase activity using the ANS sensitive TNBC cell lines MDA-MB-468 and MDA-MB-231. The MDA-MB-468 and MDA-MB-231 cell lines were treated with 1μM of each ANS analogue and cells were incubated for 48 hours, after this time-point cells were either harvested for immunoblotting or the alamar blue assay was performed. ANS obtained from Sigma-Aldrich was used in parallel as a positive control. Both cell lines were found to be comparably sensitive to the same ANS analogues (Figure 5.16A and B). Only one of the twelve compounds EMR-H380, which is synthetically manufactured ANS, induced a statistically significant decrease in cellular reductase activity, which was comparable to commercially obtained ANS. Synthetic ANS (EMR-H380), induced JNK phosphorylation with equal effectiveness as commercially obtained ANS. Two of the remaining "inducers" of the JNK pathway, EMR-F290 and IAI-202 were found not to decrease cellular reductase activity. However, only one of the two "inducers" of the JNK pathway, EMR-F290, induced detectable JNK phosphorylation (Figure 5.16C). Interestingly, the ANS analogue IAI-198 classified by its inability to induce JNK phosphorylation, induced a statistically significant decrease in cellular reductase activity in both the MDA-MB-468 and MDA-MB-231 cell lines and this effect was comparable to synthetic ANS (EMR-H380) and commercially obtained ANS. Indeed, following immunoblotting it was verified that IAI-198 did not induce detectable JNK phosphorylation (Figure 5.16C). It would be interesting to further characterise the mechanism of action of IAI-198 and to determine if this compound can bind to the ribosome and inhibit protein synthesis similar to ANS.

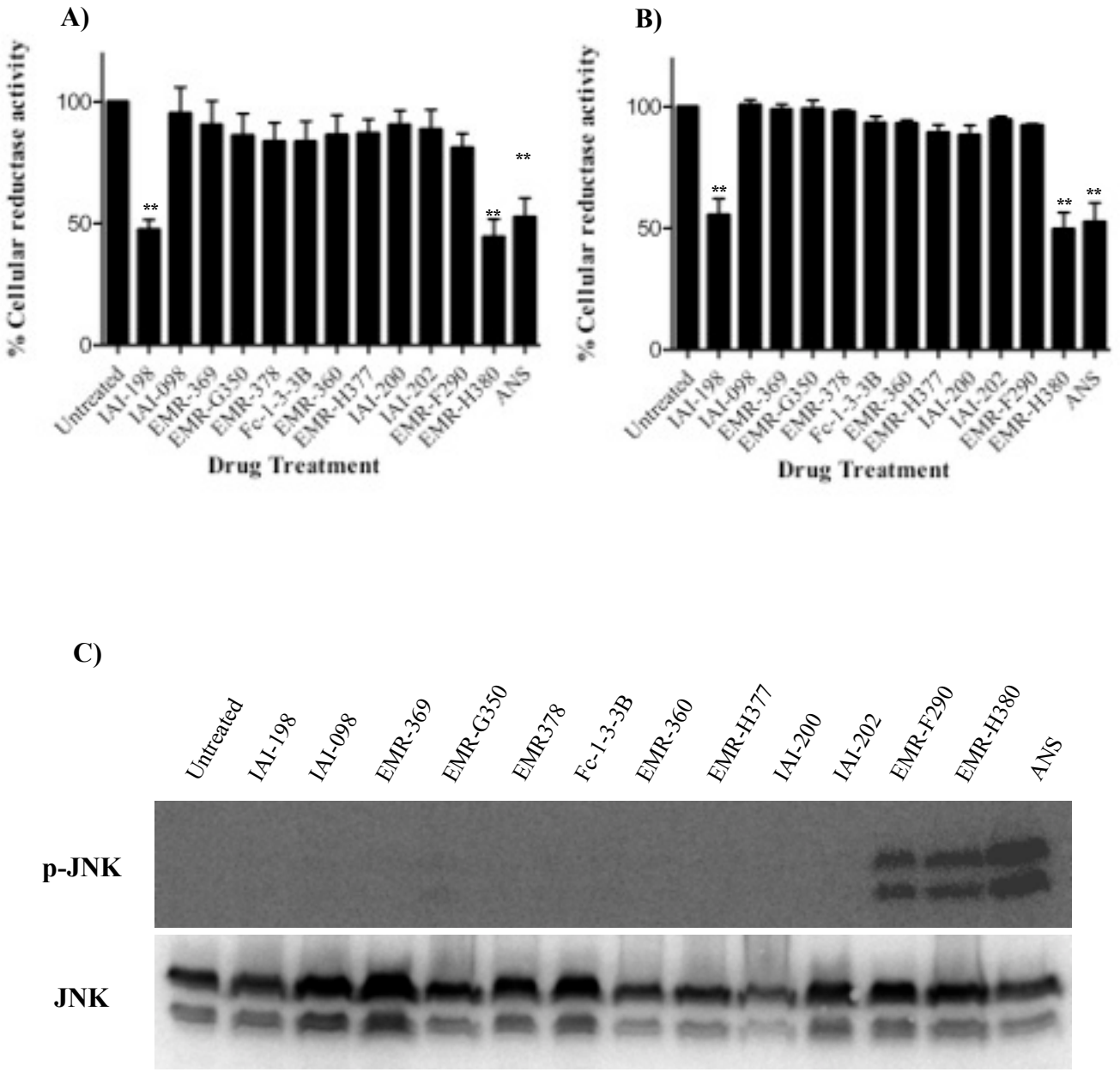
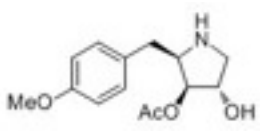


Figure 5.16 - Testing ANS analogues classified based on their ability to activate the JNK pathway at decreasing cellular reductase activity - A) MDA-MB-468 and **B)** MDA-MB-231 TNBC cell lines were screened using the twelve ANS analogues. Similar results were obtained on both cell lines, with only two compounds found to decrease cellular reductase activity; IAI-198 which is reported not to induce phosphorylation of JNK and synthetic ANS (EMR-H380), which is a potent inducer of JNK phosphorylation. **C)** MDA-MB-468 cells were treated for 6 hours with ANS analogues at a concentration of 10μM. Only EMR-F290, the synthetic ANS EMR-H380 and ANS from Sigma Aldrich induced phosphorylation of JNK. (Data shown n=3 ± SEM, one way ANOVA followed by Tukeys post hoc test, **P<0.01).

Classification: Strong JNK induction

Verification: Strong JNK induction

Inhibition of cellular reductase activity: Yes



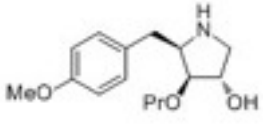
EMR-380

Molecular weight: 265.30

Classification: Strong JNK induction

Verification: Strong JNK induction

Inhibition of cellular reductase activity: No



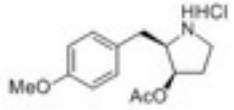
EMR-290

Molecular weight: 265.35

Classification: Strong JNK induction

Verification: Strong JNK induction

Inhibition of cellular reductase activity: No



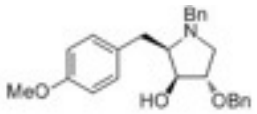
IAI-202

Molecular weight: 285.77

Classification: Moderate JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



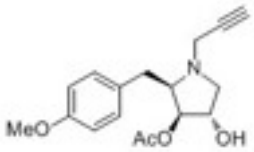
EMR-377

Molecular weight: 403.51

Classification: Moderate JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



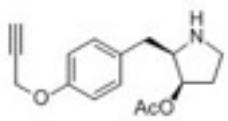
Fc-1-3-3B

Molecular weight**:** 303.35

Classification: Moderate JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



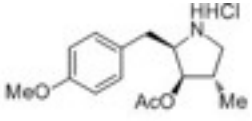
IAI-200

Molecular weight: 273.33

Classification: Moderate JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



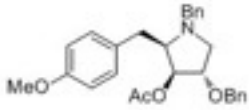
EMR-360

Molecular weight: 299.79

Classification: Moderate JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



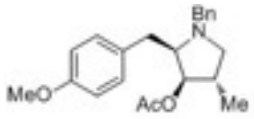
EMR-378

Molecular weight: 445.55

Classification: No JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



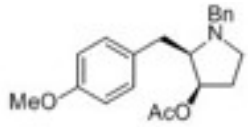
EMR-359

Molecular weight: 353.45

Classification: No JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



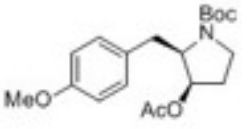
EMR-350

Molecular weight: 339.43

Classification: No JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: Yes



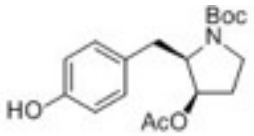
IAI-198

Molecular weight: 349.42

Classification: No JNK induction

Verification: No JNK induction

Inhibition of cellular reductase activity: No



IAI-098

Molecular weight: 355.39

Figure 5.17 - Structure and classification of the ANS analogues - The structural modification of each ANS analogue used is outlined. The classification and subsequent verification of ANS analogues including their ability to induce JNK phosphorylation and decrease cellular reductase activity are outlined. The compound EMR-380 is synthetically manufactured ANS and exhibited a profile exactly the same as commercially obtained ANS

5.3 Discussion

5.3.1 ANS displays selectivity towards certain cancer cell lines and is more effective than CHX

In the present study, ANS was identified and subsequently validated in a secondary screen as the most effective compound at decreasing cellular reductase activity in the MDA-MB-468 and MDA16 TNBC cell lines. Further characterising the spectrum of this effect identified that the MDA-MB-231 TNBC cell line was equally sensitive to ANS-mediated decrease in cellular reductase activity. However, ANS also decreased cellular reductase activity of the non-breast cancer cell line MCF10a and the prostate cancer cell lines DU145 and 22Rv1. The DU145 and 22Rv1 prostate cancer cell lines are androgen insensitive and this type of prostate cancer similar to TNBC currently do not have an effective or selective therapy. Thus, it would be interesting to further characterise what confers sensitivity of these prostate cancer cell lines to ANS and testing if the efficacy of ANS seen *in vitro* is replicated *in vivo* using preclinical syngenic and xenograft models. The ability of ANS to significantly decrease the cellular reductase activity of the non-cancer cell line MCF10a within the concentration range that is responsible for its cytotoxic effects against the sensitive cancer cell lines was concerning. However, following the identification that ANS has been successfully administered in a number of preclinical mouse models, with no reported toxicity issues, it is likely that ANS can be well tolerated in humans (Liu et al., 2009, Ruller et al., 1999). Additionally, the MCF10a cell line originates from a benin neoplasm of the basal epithelium similar to the MDA-MB-468 and MDA-MB-231 cell lines. Therefore, despite being considered to represent a “normal” cell line it is not normal. The ability of another protein synthesis inhibitor CHX, which inhibits protein synthesis at the elongation stage (Iordanov et al., 1997), to affect cellular reductase activity and colony formation was tested. ANS was found to significantly inhibit colony formation of the MDA-MB-468 cell line with an EC_{50} of 1μM. CHX displayed a similar effectiveness at decreasing cellular reductase activity as ANS. However, the ability of CHX to inhibit colony formation was significantly less effective with only a 20% decrease in colony formation observed at a concentration of 30μM. The lack of comparable effectiveness between ANS and CHX to inhibit colony formation, suggests that not all protein synthesis inhibitors are equally effective in their ability to effect the growth of cancer cell lines *in vitro*. CHX is well characterised to induce reversible cell cycle arrest at the G_2 phase (Verbin, 1967), which would contribute to the decrease in cellular reductase activity detected by the alamar blue assay and would explain the failure of CHX to decrease colony formation as effectively as ANS.

In contrast, the effectiveness of ANS to decrease cellular reductase activity and colony formation may be due to its ability to induce ribotoxic stress. Ribotoxic stress occurs when a compound inhibits protein synthesis with concurrent induction of stress response signalling via JNK or p38 resulting in cell death (Iordanov et al., 1997). Thus, while both ANS and CHX inhibit protein synthesis, it appears that there are some fundamental differences in the way cancer cells interpret the stress induced by each compound. This in turn is likely to effect the ability of these compounds to decrease cell viability and induce cell death.

5.3.2 Caspase-dependent apoptosis is not the main mode of cell death induced by ANS in the MDA-MB-468 TNBC cell line

The main mode of cell death induced by ANS reported in the literature is caspase-dependent apoptosis and occurs as a result of ribotoxic stress i.e. inhibition of protein synthesis and subsequent induction of JNK and/or p38 phosphorylation (Iordanov et al., 1997, Ouyang et al., 2005). Apoptosis has been reported to occur in the lymphoma cancer cell line U937, where ANS concentrations of 0.1μM induce significant DNA fragmentation. At a concentration of 1μM, ANS was shown to increase the percentage of cells with phosphatidyl serine externalisation, with a concurrent increase in percentage of cells with mitochondrial membrane depolarisation, all markers of apoptotic cell death (Hori et al., 2008). Low levels of caspase-3 processing was also detected when U937 cells were treated with 1μM ANS for four hours (Hori et al., 2008). ANS has been shown to decrease cellular reductase activity in the breast cancer cell line MCF-7 (Liu et al., 2009) and has been suggested to induce apoptosis in MCF-7's as evidenced by cleavage of the known caspase-3 substrate poly (ADP-ribose) polymerase (PARP) (Mingo-Sion et al., 2004). PARP is an endonuclease that when activated during apoptosis, contributes to the DNA fragmentation process. ANS has also been reported to induce ribotoxic stress and subsequent apoptosis as evidenced by an increase in Annexin-V positive staining in the colorectal cancer cell lines HCT-8 and HCT-116 (Yang et al., 2009).

Treating the MDA-MB-468 cell line with 1μM ANS in the present study induced significant depolarisation of mitochondrial membranes, chromatin condensation and low levels of caspase-3 processing and activity. Indeed the levels of ANS induced caspase-3 processing were so small that in order to detect it, VP16 induced caspase-3 processing had to be significantly over-saturated.

In agreement with the immunoblot findings, ANS induced only a four fold increase in caspase-3 like activity in the MDA-MB-468 cell line. In comparison, VP16 was capable of inducing up to a nineteen fold increase in caspase-3 like activity (data not shown). Co-treating ANS with 10μM QVD-OPh failed to protect the MDA-MB-468 cell line from the ANS-induced decrease in cellular reductase activity, cell number and colony formation. In contrast, 10μM QVD-OPh inhibited chromatin condensation induced by ANS. Thus, these data illustrate that ANS can induce caspase-dependent effects, which can be blocked by QVD-OPh in the MDA-MB-468 cell line, but inhibition of caspases is not sufficient to protect cells from ANS induced cell death.

The findings in this thesis are in conflict with reported literature, where caspase-dependent apoptosis is the only reported mode of cell death induced by ANS *in vitro*. Treatment of the murine fibroblast cell line AKR-2B, with 10μM ANS, induced caspase-3 like activity and a concurrent induction of DNA fragmentation and cleavage of rRNA (Hoppe et al., 2002). Interestingly, however ANS did not induce mitochondrial membrane depolarisation in the AKR-2B cell line, as was observed here in the MDA-MB-468 cell line (Hoppe et al., 2002). ANS induced DNA fragmentation in the lymphoma cancer cell line S49, which was inhibited by transfecting the anti-apoptotic protein BCL-2 (King et al., 2000). ANS also induced cleavage of the cellular caspase-3 substrate PARP in the breast cancer cell line MCF-7, which was blocked by the JNK inhibitor SP600125 (Mingo-Sion et al., 2004). While many findings report ANS inducing caspase activity, processing or cleavage of cellular caspase substrates that can be inhibited by co-treatment with caspase inhibitors or over-expressing anti-apoptotic proteins, one of the major limitations in these studies is the lack of complementary cell viability and cell survival assays to measure the functionality of caspase inhibition. In the present study, I have illustrated that ANS can induce caspase-dependent effects, loss of mitochondrial membrane potential and chromatin condensation, which can be inhibited by using the QVD-OPh, as well as caspase activation and processing. However, inhibiting these caspase-dependent effects failed to protect ANS-treated MDA-MB-468 cells from dying as verified by alamar blue, trypan blue and colony formation assays. It is possible that ANS is inducing caspase-independent apoptosis. Is is clear that ANS induces mitochondrial membrane depolarisation, illustrating damage to the mitochondria. Apoptosis-inducing factor (AIF), is a mitochondrial protein that can trigger the release of cytochrome c from the mitochondria, as well as inducing caspase-independent DNA fragmentation (Lorenzo et al., 1999). Blockade of caspase activity is not expected to protect cells from AIF induced cell death.

An additional possibility, is that ANS is inducing a non-apoptotic form of cell death. What is unclear at present, is whether ANS is inducing a particular form of cell death, such as apoptosis and the mode of cell death subsequently changes to an alternative mode of caspase-independent cell death when caspases are inhibited (Figure 5.18A). Alternatively, ANS may be inducing multiple cell death pathways in parallel (Figure 5.18B) such as apoptosis, caspase-independent apoptosis and/or a non-apoptotic cell death pathway. Thus, blockade of caspase activity is unable to protect the cells following ANS treatment.

Figure 5.18 - Schematic representation illustrating how ANS may induce multiple modes of cell death - A) Normal cells (solid circle), treated with ANS undergo a form of cell death, such as apoptosis (solid square) and subsequently undergo a secondary mode of cell death such as caspase-independent apoptosis or non-apoptotic cell death (solid triangle). **B)** Alternatively, normal cells (solid circle) treated with ANS undergo multiple modes of cell death simultaneously, such as apoptosis (solid square), caspase-independent apoptosis (solid triangle) and non-apoptotic cell death (solid star).

5.3.3 ANS treated MDA-MB-468 cells exhibit autophagic-like and apoptotic morphologies by TEM

Characterising the morphology of a dying cell can be utilised to determine the mode of cell death occurring in response to a particular stress such as a chemotherpeutic. Apoptotic cell death is one of the most well defined modes of cell death. Cells undergoing apoptosis visualised using TEM, display conserved morphologies such as cell shrinkage, chromatin condensation, abnormal mitochondria which appear electron dense and organised dismantling of the cell, evidenced by membrane blebbing, which allows for the compartmentalisation of intracellular components (Arbustini et al., 2008, Hayakawa et al., 2002). Autophagy is a cellular process that contributes to cell survival when cells are cultured under sub optimal growth conditions. Autophagy allows for the recycling of intracellular components and thereby generating energy to keep the cell alive until more favourable growth conditions are encountered (Mizushima, 2007, Roy and Debnath, 2010). The role of autophagy in cancer is conflicting with some studies reporting pro-survival and others pro-death effects.

From an ultrastructure perspective, autophagy can be characterised by vacuolisation of the cytoplasm called autophagosomes, which often contain intracellular components with aggregation of lysosomal membranes. When autophagosomal and lysosomal structures amalgamate, they form an autophagolysosomes, which causes the intracellular degradation of organelles and other components (Arbustini et al., 2008, Fengsrud et al., 1995).

ANS has been exclusively reported to induce apoptosis both dependently and independently of the stress activated kinase pathways JNK and p38 (Bennett et al., 2001, Eminel et al., 2004, Schwabe et al., 2004, Hoppe et al., 2002). The ultrastructural morphologies induced in response to ANS treatment in the MDA-MB-468 TNBC cell line identified and reported in this thesis represents a novel approach to identify the mode of cell death induced by ANS. The ultrastructure of ANS treated cells has never before been reported and thus, the present study offers an exciting addition to the existing knowledge. ANS has been shown to induce cell death in the TNBC cell line MDA-MB-468, with caspase-dependent chromatin condensation and mitochondrial membrane depolarisation. In addition, TEM ultrastructure images illustrate that some ANS-treated cells undergo apoptosis and share morphological similarities to VP16 treated cells. However, despite inhibition of caspase activity and preventing chromatin condensation, QVD-OPh failed to protect ANS-treated cells from undergoing cell death.

The ultrastructure of the majority of ANS-treated cells appear to have irregular or speckled chromatin condensation, swollen mitochondria, cytoplasmic vacuolisation (which appear to have intracellular inclusions), cell shrinkage and aggregation of lysosomes. Interestingly, co-treating MDA-MB-468 cells with ANS and the caspase inhibitor QVD-OPh, reversed ANS-induced chromatin condensation and seemed to reduce cell shrinkage as co-treated cells appear larger. Additionally, ANS-induced cytoplasmic vacuoles appeared smaller in the presence of the caspase inhibitor, but there was also an increase in the aggregation of lysosome like membranes.

These data illustrate, that ANS treatment induces morphologies consistent with apoptosis, due to the similarities with VP16 treated cells. The novel finding in this thesis is that ANS induces cell death that is caspase-independent and shares significant similarities with autophagy. Caspase-independent cell death induced by ANS has never been reported before. However, it is impossible to determine from TEM whether these cells are undergoing autophagic-like cell death.

Without molecular testing, the role of these autophagy-like cells and their contribution to ANS induced cell death or attempted cell survival in the presence of ANS-induced cellular stress, is impossible to conclude.

Most significantly, and with particular relevance to the effectiveness of ANS as a potential anticancer agent for the treatment of TNBC, is the ability of ANS to induce two cell death pathways in parallel. Most relevant is the disassociation between the pathways, as blockade of caspase activity using the caspase inhibitor QVD-OPh prevents ANS-induced apoptosis but failed to protect ANS-treated cells from undergoing cell death. This is functionally important as acquisition of resistance by perturbation in molecular components of the apoptotic cell death pathway, such as over-expression of Bcl-xL and p53 mutation frequently occurs in cancers (Wei et al., 2012, Aas et al., 1996). The ability of ANS to activate an alternative cell death pathway in parallel, will likely increase the responsiveness of a cancer expressing these clinically relevant drug resistant markers.
The cross-talk between apoptosis and autophagy does not always result in execution of the cell, as autophagy has been shown to protect cells from apoptotic cell death (Roy and Debnath, 2010). Depolarisation of the mitochondria in response to metabolic stress following drug treatment, normally results in generation of reactive oxygen species (ROS), that induces DNA damage resulting in apoptosis. However, tumour cells can utilise autophagy to scavenge depolarised mitochondria, a process known as mitophagy, which prevents the generation of ROS and thereby protects cells from induction of DNA damage and subsequent apoptosis (Karantza-Wadsworth et al., 2007, Yang et al., 2006). Interestingly, ANS has been reported to inhibit the induction of autophagy following serum starvation in the mammalian HEK-293 cell line. Inhibition of autophagy by ANS was attributed to the ability of ANS to induce phosphorylation and activation of the p38 pathway which regulates mAtg9, a key protein in the formation of autophagosomes (Webber and Tooze, 2010). Despite this finding ANS has not been reported to inhibit or induce autophagy using cancer cell lines *in vitro*. It would be interesting to determine if ANS induces similar apoptosis and autophagic-like ultrastructural changes in other ANS-sensitive cell lines identified in this thesis, such as the MDA-MB-231 breast cancer and the 22Rv1 and DU-145 prostate cancer cell lines. This will address whether the ability of ANS to induce autophagic-like and apoptotic morphologies is a cell line specific effect.

5.3.4 ANS induces phosphorylation of JNK but not p38 and inhibitors of these kinases does not protect against ANS mediated decrease in cellular reductase activity

In agreement with published literature, ANS was found to induce phosphorylation of both JNK isoforms, p46 and p54, in the ANS sensitive MDA-MB-468 and ANS insensitive Hs578t cell lines over a six hour time-course. In contrast to many reports, ANS failed to induce phosphorylation of p38. Pharmacological inhibition of JNK phosphorylation with SP600125 or CEP-11004, has previously been reported to prevent JNK phosphorylation and subsequently protect cells from JNK-mediated caspase activation and decreased cellular reductase activity (Bennett et al., 2001, Eminel et al., 2004, Schwabe et al., 2004, Xia et al., 2007). In the present study, SP600125 was found to significantly decrease ANS induced JNK phosphorylation as determined by immunoblotting. However, this effect was not paralleled with protection of the MDA-MB-468 cell line from ANS-mediated decrease in cellular reductase activity or colony formation. This data either suggest, that the degree of JNK phosphorylation inhibited by SP600125 is not sufficient to fully inhibit its functionality or alternatively, JNK phosphorylation is a consequence of ANS treatment and is not required for ANS's effect in the MDA-MB-468 cell line. In agreement with the latter conclusion, inhibition of ANS-induced JNK phosphorylation using SP600125 in the murine fibroblast cell line did not protect cells from the ANS-induced decrease in cell number (Hoppe et al., 2002). In addition, Hoppe et al, concluded that neither JNK nor p38 phosphorylation play a role in ANS-induced apoptosis in the AKR-2B murine fibroblast cell line. As was expected, due to the inability of ANS to induce phosphorylation of p38 in the MDA-MB-468 and Hs578t cell lines, pharmacological inhibition of p38 using SB203580 did not protect the MDA-MB-468 cell line against the ANS-induced decrease in cellular reductase activity.

5.3.4.1 ANS analogues classified by there ability to activate the JNK pathway illustrate that JNK activation does not correlate ANS induced cell death

In order to determine the role, if any, that the JNK pathway plays in ANS induced cell death, twelve ANS analogues classified by their ability or inability to induce JNK phosphorylation (Rosser et al., 2004) and subsequent effect on cellular reductase activity was tested against the MDA-MB-468 and MDA-MB-231 cell lines.

Only one of the two compounds classified as inducers of JNK phosphorylation, EMR-F290 was confirmed to induce JNK phosphorylation in the MDA-MB-468 cell line, but failed to decrease cellular reductase activity in either the MDA-MB-468 or MDA-MB-231 cell lines. IAI-202, the second compound classified as an inducer of JNK phosphorylation, did not induce detectable levels of JNK phosphorylation and did not decrease cellular reductase activity. Synthetic ANS was illustrated, as expected, to induced JNK phosphorylation and decreased cellular reductase activity with equal effectiveness as commercially available ANS. The most interesting finding however, was that IAI-198, an ANS analogue classified as a non-inducer of JNK phosphorylation, decreased cellular reductase activity in both the MDA-MB-468 and MDA-MB-231 cell lines to a similar level as ANS. In addition, IAI-198 was verified not to induce detectable levels of JNK phosphorylation. Thus, the inability of a JNK inhibitor to protect cells against ANS induced decrease in cellular reductase activity, in addition to the data generated using the ANS analogues, suggests that at least in the MDA-MB-468 cell line, induction of the JNK stress response pathway does not correlate with ANS induced cell death.

5.3.6 MCL-1 does not determine cell fate in response to ANS

The two *bona fide* protein synthesis inhibitors ANS and CHX were shown to decrease the expression of MCL-1, a short half-life anti-apoptotic protein after just six hours in the ANS sensitive MDA-MB-468 and the less sensitive Hs578t TNBC cell lines. This data correlates with published literature relating to the protein synthesis inhibition activities of these compounds (Iordanov et al., 1997, Barbacid and Vazquez, 1974) and suggests that in the present study protein synthesis is inhibited by ANS. The ability of ANS to inhibit protein synthesis in cells that are both sensitive and insensitive is an interesting finding.

It suggests that the fate of cancer cells in response to ANS treatment is not directly determined by global protein synthesis inhibition, but relies on how the cell interprets the stress signal induced by ANS.

Here, induction of JNK phosphorylation has been shown not to correlate with ANS induced cell death in the MDA-MB-468 cell line, as well as ANS-induced cell death observed in the AKR-2B murine fibroblast cell line (Hoppe et al., 2002). Thus, what is the molecular component(s) that signals for a cell to survive or die in response to ANS treatment? Cancer cells often have elevated levels of pro-survival factors, which they depend on for survival as they cancel out the presence of abnormally large levels of pro-apoptotic signals (Weinstein and Joe, 2008).

Inhibition of a cancer cells achilles heel, has been reported to induce oncogenic shock, resulting in the rapid demise of the cancer cell (Sharma and Settleman, 2010). Indeed, this has been the basis of targeted drug discovery. The short half-life anti-apoptotic protein MCL-1 is known to be over-expressed in a variety of human tumours (Krajewska et al., 1996a, Krajewska et al., 1996b) and is associated with tumour development and resistance to therapy (Chen et al., 2007). Global transcriptional inhibitors such as anthracyclines have recently been shown to selectively effect MCL-1 expression due to its short half-life. It has been suggested that in anthracycline-sensitive cancer cells, inhibiting MCL-1 expression is one of the main mechanisms by which anthracyclines induce apoptosis (Wei et al., 2012). Interestingly, elevated BCL-xL expression has been illustrated to confer resistance to compounds that decrease the expression of MCL-1 (Wei et al., 2012). This finding offered a potential explanation as to how some cell lines in the present study display sensitivity to ANS, while other cells were less sensitive. However, siRNA knockdown of MCL-1 expression in the ANS sensitive MDA-MB-468 cell line, failed to mimic the decrease in cellular reductase activity observed with ANS. This illustrates that MCL-1 does not confer sensitivity to ANS nor does it play a role in ANS-induced cell death.

At present, not enough mechanistic information for ANS has been elucidated and as such this agent is not yet suitable for translation as an anticancer agent. However, based on the current findings within this thesis, ANS appears to have significant potential and it is imperative that ANS's molecular target or indeed a biomarker of sensitivity to ANS is identified in order to predict how different cancer cell lines are likely to respond to ANS treatment. Identification of either ANS's target or a biomarker will increase the attractiveness of this compound for repurposing.

Chapter 6
Final Discussion

6.1 Screening and repurposing

One aim of this project was to screen the JHCCL against the MDR1 over-expressing MDA16 TNBC cell line, in an attempt to identify novel compounds that caused collateral sensitivity. Indeed, four compounds were identified that caused collateral sensitivity, three related antimetabolites cladribine, cytarabine and gemcitabine which have previously been reported to cause collateral sensitivity in a number of cancer cell lines that over-express MDR1 or MRP1. Thus, the novelty of the finding that these compounds caused collateral sensitivity no longer applies. The final compound that was identified to cause collateral sensitivity was the antineoplastic agent dacarbazine. However, this compound did not effectively decrease cellular reductase activity and was therefore not suitable for further characterisation. Due to the lack of compounds that effectively caused collateral sensitivity, the second aim as outlined in the decision tree (Figure 3.5), was implemented to identify compound(s) for further characterisation. The second aim was to identify compounds that exhibited novel activities against cancer cell lines *in vitro* and compounds that exhibited effectiveness in their ability to decrease cellular reductase activity. Following secondary screening, only the antifungal CPX and the antibiotic ANS were identified to possess both novelty and effectiveness in their ability to decrease cell viability against the MDA-MB-468 and MDA16 TNBC cell lines. Due to this, both compounds were selected for further characterisation in an attempt to identify the spectrum of their effect, the mode of cell death induced and the molecular target required for the ability of these compounds to decrease cell viability. It has been illustrated here, that selection of an appropriate assay significantly contributes to the identification of compounds with novel cytotoxic activities against cancer cell lines. Importantly, it is evident that repurposing is an attractive approach for the identification of compounds with novel activities and that *in vitro* identification and validation is a rapid process. The ability to translate a hit compound following its *in vitro* characterisation is easier when compared to the translation process for an NCE. From the screen of the JHCCL, there were other compounds that displayed effectiveness in their ability to decrease cellular reductase activity of the TNBC cell lines MDA-MB-468 and the MDR daughter cell line MDA16. This form of unbiased screening could be useful for identification of compounds that ultimately add to the current repertoire of anticancer agents, not just for TNBC but for a variety of other cancer types. However, caution must be taken as favourable results *in vitro* do not necessarily guarantee that a compound will be successfully repurposed.

One of the major attractions with repurposing as an alternative approach for the identification of compounds that possess potential anticancer activities, is that in theory the cost of repurposing should be significantly less than the cost of developing and marketing a NCE.

6.1.1 Pharmacoeconomics

The research and development required for the production of rationally designed therapies such as the monoclonal antibodies, bevacizumab and trastuzumab, and targeted kinase inhibitors, imatinib, is significantly greater compared to conventional drug development. This increased complexity is associated with an increase in the cost of manufacturing targeted therapies, a cost subsequently passed on to the patient. Treatment of HER2 over-expressing breast cancers with the monoclonal antibody, trastuzumab in combination with conventional cytotoxic chemotherapeutic agents such as doxorubicin, docetaxel or carboplatin costs approximately €50,000 per year per patient. Additional costs to consider include characterisation of disease type, mastectomies, regular check-ups and adjuvant hormone therapy which can increase the cost by approximately €4,000 per year (Liberato et al., 2007, Neyt et al., 2006). Bevacizumab, a monoclonal antibody that targets VEGF resulting in the inhibit of angiogenesis is another targeted anticancer therapy. Bevacizumab is currently used to treat both lung and colorectal cancers. Bevacizumab is commonly administered in combination with 5-FU and folinic acid for the treatment of metastatic colorectal cancer at a cost of approximately €100,000 per year (Shiroiwa et al., 2007). These costs massively eclipse the cost of conventional chemotherapeutics such as doxorubicin and docetaxel, which are between €10,000 and €20,000 per year. The exorbitant cost of target therapies, is most likely attributable to the lack of equally efficient alternatives. Despite this however, less than 35% of HER2 over-expressing metastatic breast cancers respond to trastuzumab therapy in the first instance and development of resistance is a major problem. The lack of initial response observed to targeted therapies and the development of resistance, illustrates the importance of implementing a more accurate method of patient stratification, to identify tumours most likely to respond to a particular treatment regimen. Currently, the ability to perform gene expression profiling on patient tumour samples is not achievable in the clinic as it is too time consuming and costly. One of the foreseeable disadvantages of more stringent patient stratification is the likely knock-on price increase owing to the overall reduction in treatable patients. As mentioned above, one of the advantages discussed in a number of repurposing related articles and reviews is the ease of manufacturing “approved drugs”.

Identification of a novel anticancer activity in an "approved drug" is an attractive alternative to novel drug discovery for pharmaceutical companies, as these compounds have been mass produced in the past and in some cases at present. Thus, one would expect that these compounds should be significantly less expensive compared to trastuzumab or bevacizumab. Alas, this is not the case. Thalidomide was a drug with limited medical value and commercial value before it was repurposed for the treatment of refractory multiple myeloma. The only reported medical use of thalidomide was for the treatment of erythema nodosum laprosum, a painful inflammatory disease associated with HIV. Interestingly the cost of thalidomide has risen dramatically between 2000 and 2010 going from $98.40 per prescription of thirty 50mg capsules to $6,000 per prescription of thirty 50mg capsules (Hemus, 2008, Minuk et al., 2010), thus illustrating that thalidomide is as expensive as trastuzumab. This cost of thalidomide is clearly not associated with optimisation of manufacturing, as this process have been well established since the 1960s. Instead, the cost can be attributed to a lack of effective alternative therapies for the treatment of refractory multiple myeloma and covering the cost of clinical trials testing thalidomide as an anticancer agent (Berenson, 2005). Lenalidomide, an analogue of thalidomide which is reportedly more potent and associated with less side effects, is replacing thalidomide for the treatment of multiple myeloma. This newer and more potent compound is significantly more expensive than thalidomide with monthly prescriptions of $8,000-$10,000 (Beacon, 2011). As thalidomide and lenalidomide are the only two novel anticancer agents repurposed over the last decade it is unreasonable to assume that future repurposed compounds will be associated with the same level of price inflation. However, if these price increases are associated with all future repurposed drugs, one must ask who are we as researchers attempting to cure, as the cost of these therapies suggests only the wealthy will be able to afford these drugs (Beacon, 2011). This also begs the questions, why and how are these compounds, which have been manufactured and sold at a significantly lower price for the treatment of a different disease, can become as expensive as rationally designed targeted therapies such as trastuzumab and bevacizumab. A recent topic of interest in many scientific journals is the inability of pharmaceutical companies to match supply with market demand resulting in a shortage of anticancer agents. Interestingly, this shortage is limited to the more conventional cytotoxic chemotherapeutics such as doxorubicin and etoposide. Considering the length of time that these compounds have been in the pipeline, this sudden shortage is clearly not attributable to difficulties in the manufacturing process. The most likely explanation is that the big pharmaceutical companies are making readily available the newer, more expensive anticancer agents and phasing out the older therapies.

6.2 Collateral sensitivity

One aim of this study was to identify a compound(s) that caused collateral sensitivity i.e. the drug resistant TNBC cell line MDA16 would be more sensitive to the effects of a drug than the MDA-MB-468 cell line. The rational behind attempting to identify a collateral sensitivity causing compound, stems from the development of resistance *in vivo* and the subsequent need for developing compounds that can overcome this resistance and induce tumour cell death. There are numerous reports of compounds causing collateral sensitivity *in vitro,* such as the antimetabolite agents cytarabine and gemcitabine in lung, prostate and melanoma cancer cell lines that over-express either MDR1 or MPR1 (Jensen et al., 1997, Bergman et al., 2001, Bergman et al., 2003). Despite the reports of collateral sensitivity *in vitro*, it has not been reported to occur *in vivo* (Hall et al., 2009). Collateral sensitivity is an interesting observation, as it has changed how researchers think about drug resistance *in vitro* and *in vivo*. If collateral sensitivity is not integrated into our assessment of drug resistance, resistance very often it implies that killing a drug-resistant cancer cell is almost impossible. The concept of collateral sensitivity changes the definition of resistance, because now it is apparent that the molecular changes conferring resistance, inherently make the "resistant" cancer cell more sensitive to a different drug. Now the difficulty arises in identifying the cancer cells "achilles heel". Once the cancer cells "achilles heel" is identified, this will allow for the development of therapies capable of targeting and thus, killing the resistant cancer cell.

From a clinical perspective, compounds like ANS, which illustrate effectiveness against both sensitive and resistant cancer cell lines, such as MDA-MB-468 and MDA16*,* are more attractive than a compounds like cladribine, which is effective only against the MDR1 over-expressing, resistant MDA16 cancer cell line. It is logical to assume based on the *in vitro* data, that if ANS was approved for the treatment of TNBC, that it would be more effective as part of the current treatment regimen, than the collateral sensitivity causing agent cladribine. Furthermore, the selection of an appropriate collateral sensitivity agent in the clinic is complicated by the use of combination therapy. This could lead to multi-factorial drug selection, reducing the effectiveness of the agent in a clinically setting.

In summary, *in vitro* collateral sensitivity is intellectually attractive and can aid in the identification of a cellular target that is a resistant cancer cells achilles heel.

From a clinical viewpoint, identification of a compound capable of killing sensitive and resistant cancer cells is more attractive than a compound that causes collateral sensitivity, as this compound is more likely to augment the effects of current cancer chemotherapeutics.

6.3 Identification and repurposing of CPX

During the validation and characterisation of CPX against the MDA-MB-468 and MDA16 TNBC cell lines, it was reported that CPX was screened against a panel of leukemic and lymphoma cancer cell lines *in vitro* and effectively decreased cell viability of all the cell lines tested (Eberhard et al., 2009). Subsequently, CPX was shown to decrease tumour growth rate against a panel of preclinical haematological cancer xenograft models (Eberhard et al., 2009). Due the the effectiveness of CPX *in vitro* and *in vivo* against haematological cancer cell lines, a clinical trial was established testing the effect of CPX against a range of human haematological malignancies. This clinical trial is set to finish in October 2012 and the outcome will determine if CPX is going to be successfully repurposed for the treatment of certain haematological cancers. Additionally, in this thesis CPX selectively decreased cellular reductase activity in a number of cancer cell lines. This correlated with CPX inducing apoptosis in the MDA-MB-468 and MDR-TNBC MDA16 cell lines as evidenced by caspase-3 processing and activation. Co-incident with these findings, CPX was reported to induce apoptosis and to inhibit tumour growth of the TNBC cell line MDA-MB-231 using a preclinical xenograft model (Zhou et al., 2010). Thus, the novelty of CPX to decrease cellular reductase activity and induce apoptosis *in vitro* against a panel of cancer cell lines has been lost. Due to this the effects elicited *in vitro* by ANS was tested and given precedence.

6.4 ANS's molecular target and ribotoxic stress

ANS has been reported to inhibit protein synthesis for almost half a century, and was initially reported to inhibit protein synthesis in HeLa cells, rabbit reticulocytes and in cell free extracts (Grollman, 1967). ANS is known to preferentially bind to the 60S subunit of active 80S ribosomes, where it inhibits peptidyl transferase activity, thereby shutting down protein synthesis (Barbacid and Vazquez, 1974). The affinity of the interaction between ANS and the ribosome is very strong with a Kd of $1.6x10^{-6}$M (Barbacid and Vazquez, 1974, Janssen, 1977). The crystal structure of ribosome bound ANS, has been elucidated and it has been shown that only one molecule of ANS binds per ribosome (Hansen et al., 2003, Bulkley et al., 2010).

It is now known the the *p*-methoxyphenyl group of ANS, binds to the a pocket in the A site of 80S ribosomes and this is how it inhibits peptidyl transferase activity (Hansen et al., 2003). It has been shown *in vitro,* using *Haloarcula marismortui,* that there are two main mutation types that confer resistance to ANS. Mutation of the nucleotides participating in ANS-ribosome binding, inhibit the ability of ANS to bind the ribosome thereby preventing inhibition of peptidyl transferase. A second type of mutation occurs in nucleotides around the ANS binding site. This alters the confirmation of the binding site preventing ANS's access and thereby blocks the ability of ANS to inhibit peptidyl transferase (Blaha et al., 2008). To date no such mutations have been reported in cancer cell lines. The inability of ANS to reduce cellular reductase activity against some cancer cell lines shown here is unlikely due to ribosomal mutations, as ANS was found to decrease MCL-1 protein levels of the ANS insensitive TNBC Hs578T cell line and colorectal cancer cell line HT-29 to comparable levels as the ANS sensitive MDA-MB-468 cell line (Figure 5.13A and C). In addition to inhibiting peptidyl transferase, ANS treatment has been reported to inhibit the formation of 80S ribosomes as evidenced by the increase of 60S subunit (Janssen, 1977). In response to protein synthesis inhibition, ANS induces a stress response termed "ribotoxic stress", where the stress kinases JNK and/or p38 are activated and induce cellular demise via apoptosis (Iordanov et al., 1997, Laskin et al., 2002). Blockade of these kinases decreased caspase-3 like activity and processing as well as other caspase specific effects such as DNA fragmentation and cytochrome c release (Bennett et al., 2001, Schwabe et al., 2004, Eminel et al., 2004). This illustrates a link between induction of "ribotoxic stress", caspase activation and apoptosis

Here, ANS decreased the expression of MCL-1 an anti-apoptotic short-half life protein, in the ANS-sensitive MDA-MB-468 and ANS insensitive Hs578t TNBC and HT-29 colorectal cell lines. Another protein synthesis inhibitor CHX, decreased MCL-1 expression with similar effectiveness as ANS in both the MDA-MB-468 and Hs578t cell lines. However, despite CHX and ANS illustrating similar effectiveness in their ability to decrease cellular reductase activity, CHX was found to be significantly less effective at reducing cell number and colony formation compared to ANS. In addition, knockdown of MCL-1 levels using siRNA did not induce a similar decrease in cellular reductase activity as ANS. This data suggests that ANS affects protein synthesis as evidenced by the decreased expression of MCL-1 and thus, correlates with reported literature. However inhibition of protein synthesis *per se* nor enforced reduction of MCL-1 is sufficient for the induction of cell death.

ANS was found to induce ribotoxic stress as defined by Iordanov et al., 1997, in the ANS sensitive MDA-MB-468 and less sensitive Hs578t cell lines due to phosphorylation of the stress activated kinase JNK (Iordanov et al., 1997). Pharmacological inhibition of JNK phosphorylation induced by ANS using SP600125, did not protect the MDA-MB-468 cell line from ANS-mediated decrease in cellular reductase activity, cell number or colony formation. It is worth mentioning, that despite using 30μM of the JNK inhibitor, SP600125, ANS induced JNK phosphorylation was not completely inhibited. Higher concentrations of the SP600125 were not used, as 30μM SP600125 alone induced a statistically significant decrease in cellular reductase activity and colony formation. However, using ANS analogues classified by their ability or inability to activate the JNK pathway, illustrated that an ANS analogue capable of inducing JNK phosphorylation was incapable of decreasing cellular reductase activity. Conversely, an ANS analogue that did not induce detectable JNK phosphorylation was capable of effectively decreasing cellular reductase activity in the MDA-MB-468 cell line. These findings are in conflict with some of the published data regarding the induction of JNK phosphorylation by ANS and subsequent activation of caspases, which can be reversed using JNK inhibitors (Xia et al., 2007, Mingo-Sion et al., 2004). These data suggest that ANS induced JNK phosphorylation does not contribute to ANS induced cell death. Despite this however, the ribosome still cannot be ruled out as the cellular target of ANS, responsible for inducing cell death as ANS clearly decreases expression of MCL-1 and does induce "ribotoxic stress" signalling.

Both of these effects do not contribute to ANS-induced cell death. So how is ANS working? There are two potential explanations:

i) ANS inhibits protein synthesis and this inhibition induces a stress response signalling pathway other than what is described by the current definition of ribotoxic stress e.g. causing an imbalance in the levels of the 60S ribosomal subunit.

ii) ANS induces cell death independently of protein synthesis inhibition and ribosomes, instead affecting an as yet unidentified cellular target.

ANS has been shown to increase cellular levels of the 60S ribosomal subunit by preventing the formation of functional 80S ribosomes (Grollman, 1967). It has been reported that L11, a protein that forms part of the 60S ribosomal subunit, can negatively regulate MDM2 resulting in the stabilisation of p53 expression levels.

It has been suggested that the ability of L11 to induce stabilisation of p53, acts as a ribosomal checkpoint during ribosomal biogenesis and assembly (Zhang et al., 2003). Thus, the ability of ANS to increase the levels of the 60S ribosomal subunit may result in elevated L11 levels, activating a ribosomal checkpoint.

6.5 ANS induces cellular morphologies consistent with apoptosis and autophagy

The ability of ANS to induce two independent modes of cell death is a favourable finding, which increases the potential usefulness of this compound if it is successfully repurposed for the treatment of TNBC. In this study, it has been illustrated that ANS can induce apoptosis with classical apoptotic events occurring both molecularly, such as mitochondrial membrane depolarisation, caspase activation and caspase processing, and morphologically including cell shrinking, chromatin condensation, abnormal mitochondria and membrane blebbing. Interestingly, these effects were blocked by the caspase inhibitor QVD-OPh, however this failed to protect the cell from death illustrating that an alternative cell death pathway is activated in parallel to apoptosis and which occurs independently of caspases. As visualised by TEM, ANS treated cells that display the non-apoptotic morphology, share morphological characteristics that resemble autophagy. These include formation of cytoplasmic vacuoles or autophagosomes, which appear to contain intracellular inclusions, incomplete chromatin condensation and aggregation of lysosome like membranes. It is impossible to determine if a) this autophagy-like morphology is truly autophagy and b) if this autophagy-like morphology is contributing to the cell death induced by ANS, without performing molecular based experiments.

6.6 Autophagy and cancer

Autophagy is a catabolic process, whereby intracellular components are sequestered in cytoplasmic vacuoles called autophagosomes. These autophagosomes fuse with enzyme containing lysosomes resulting in the formation of autophagolysosomes, which degrade cellular components that are recycled in the cell (Mizushima, 2007). The role of autophagy in cancer is conflicting, with both pro-survival and pro-death functions being reported. In support of its pro-survival function, it has been reported that under hypoxic conditions in cancer cell lines, stabilisation of the hypoxia inducing factor-1α (HIF-1α), can induce autophagy of mitochondria, known as mitophagy.

This protects cells from reactive oxygen species (ROS) and subsequent DNA damage, thus contributing to cell survival (Zhang et al., 2008). In agreement with this, the hypoxic core of solid tumours undergo autophagy, which contributes to cancer cell survival (Degenhardt et al., 2006). The pro-death role of autophagy in cancers has been illustrated *in vitro*. The antimetabolite gemcitabine, has been shown to induce autophagy and subsequent apoptosis in pancreatic cancer cell lines and inhibition of autophagy, significantly decreased gemcitabine induced apoptosis (Pardo et al., 2010). This suggests that autophagy promotes cancer cell death by apoptosis. In addition, autophagy has been shown to directly induce cell death. Treating the leukemic cancer cell line HL-60, with vitamin K induced both apoptosis and autophagy. Blockade of apoptosis, increased vitamin K-induced autophagic-cell death (Yokoyama et al., 2008). This has been illustrated for other compounds, such as the proteosome inhibitor MG132, which induced apoptosis and autophagy in the prostate cancer cell line PC3 (Yang et al., 2006) and arsenic tetroxide, which induced both apoptosis and autophagy in T-lymphocyte leukemic cancer cell lines (Qian et al., 2007).

It has recently been reported that a range of clinically relevant chemotherapeutics, significantly decreased tumour growth rate in a syngenic colorectal carcinoma mouse model, via the induction of autophagy but not apoptosis (Michaud et al., 2011). Syngenic models that possessed gene knockouts of key autophagy mediators, Atg5 and Atg7, were found to have significantly less inhibitory tumour growth response to chemotherapeutics (Michaud et al., 2011). Treating immunocompromised colorectal cancer xenograft models with mitoxantrone failed to reduce tumour growth. In contrast, using immunocompetent BALB/c mice as colorectal cancer xenograft models, mitoxantrone induced a significant reduction in tumour growth (Michaud et al., 2011). It was finally concluded that induction of autophagy was essential for eliciting an immunogenic response, which increases the effectiveness of an anticancer agent. Unlike autophagy, apoptosis does not induce an immunogenic response and thus, is less effective at decreasing tumour growth at least in preclinical models of cancer.

Thus, the role of autophagy in cancer is complex with both pro-survival and pro-death effect being elucidated. It appears that the effect of autophagy, whether pro-death or pro-survival, is context dependent i.e. cancer type (including mutations harboured by a particular cancer) and choice of chemotherapeutic. ANS was found to induced both apoptosis and a caspase-independent form of cell death.

TEM images of ANS treated cells, exhibit an autophagic-like morphology. Whether these cells are under going cell death or attempting to survive in the presence of ANS induced stress is uncertain. However, what is known is that a caspase-independent form of cell death is contributing to ANS's effect. As outlined in Figure 5.18, whether ANS is inducing an autophagic-like cell death with subsequent apoptosis, or inducing multiple cell death pathways in parallel, which contribute to ANS induced cell death in the MDA-MB-468 cell line is currently unknown. It would be interesting to determine if the caspase-independent mode of cell death is autophagy, caspase-independent apoptosis or indeed an as yet unclassified form of cell death.

In conclusion, I have identified a number of compounds that exhibit effectiveness at decreasing cellular reductase activity against the MDR-TNBC cell line MDA16. From the initial thirty compounds identified from the primary screen, the antibiotic ANS was the most effective. The activity of ANS, correlates with those reported in the literature, including induction of ribotoxic stress signalling, induction of caspase activation and apoptosis and inhibition of protein synthesis. However, further experimentation identified inhibition of ribotoxic stress signalling and caspase-3 activity does not protect cells from undergoing ANS-induced cell death. This data illustrates that ANS is eliciting an effect that has not been previously reported and is inducing a caspase-independent mode of cell death.

Figure 5.19 outlines the potential mechanisms by which ANS induces cell death in sensitive cancer cell lines. Its is possible that ANS's ability to bind the ribosome can induce an uncharacterised stress response pathway i.e. independent of JNK or p38, which induces both caspase dependent and independent forms of cell death. If this proves true, the definition of ribotoxic stress induced by certain translation inhibitors will need to be amended. An alternative explanation, is that ANS effects a cellular target that is not the ribosome, resulting in cell death. Identification of the molecular target, underpinning the cytotoxic activities of ANS will be essential for determining the patients most likely to respond to ANS based therapy, should ANS make it to clinical trials. In addition to identifying the cellular target, determining the effectiveness of ANS to inhibit tumour growth in preclinical models of TNBC will be essential. The ability of ANS to induce more than one cell death process in combination with its effectiveness and killing MDR-TNBC cell lines, makes it an attractive compound to further characterise and repurpose.

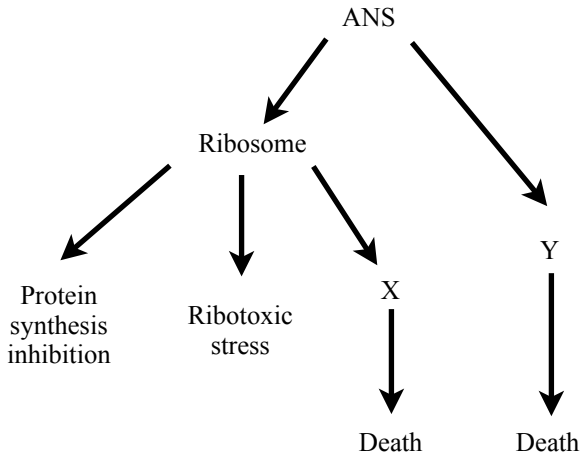


Figure 5.19 - Potential mechanisms by which ANS induces cell death - ANS has been shown to decrease the expression of MCL-1 protein and subsequently induce ribotoxic stress signalling, thus correlating with its reported activity. However, inhibition of ribotoxic stress signalling and caspase activity did not protect cell from ANS induced cell death. This suggests that ANS may bind the ribosome and induce an uncharacterised stress signalling response "X", that induced both caspase dependent and independent cell death. Alternatively, ANS may act independently of the ribosome, affect an uncharacterised cellular target "Y", which induces both caspase dependent and independent cell death.

APPENDIX

A1- Buffers and materials required for the caspase activity assay

Lysis Buffer	**Substrate Buffer**
50mM HEPES-KOH, pH7.2	40µM DEVD-afc*
5mM EGTA	50mM HEPES-KOH, pH7.2
10mM KCl	5mM EGTA
2mM $MgCl_2$	10mM KCl
2mM DTT*	2mM $MgCl_2$
10µl/ml 10% CHAPS	2mM DTT*
	1µl/ml CLAP*

Notes [*]: DTT, CLAP and DEVD-afc added fresh before use

10X PBS, pH 7.4	**CLAP protease inhibitor***
137mM NaCl	2mg/ml Chymostatin
2.7mM KCl	2mg/ml Leupeptin
100mM Na_2HPO_4	2mg/ml Antipain
2mM KH_2PO_4	2mg/ml Pepstatin A

Notes [*]: CLAP protease inhibitor is dissolved in DMSO

Material	Supplier	Product code
Potassium chloride	Sigma-Aldrich	60128
CHAPS	Sigma-Aldrich	C3023
DTT	Sigma-Aldrich	D9779
HEPES	Sigma-Aldrich	H3375
Magnesium chloride	Sigma-Aldrich	63068
Potassium hydroxide	Sigma-Aldrich	P5958
EGTA	Sigma-Aldrich	E3889
Ac-DEVD-AFC	Sigma-Aldrich	ALX-260-032-M001

A2- List of reagents required for making buffers

Material	Supplier	Product Code
Trizma Base	Sigma-Aldrich	T6066
Glycine	Sigma-Aldrich	50046
Sodium dodecyl sulfate	Sigma-Aldrich	L3771
Sodium Chloride	Sigma-Aldrich	S5886
Potassium Chloride	Sigma-Aldrich	60128
Sodium phosphate dibasic	Sigma-Aldrich	S5136
Sodium phosphate monobasic	Sigma-Aldrich	P5655
CHAPS	Sigma-Aldrich	C3023
Methanol	Sigma-Aldrich	34860
DL-Dithiothreitol	Sigma-Aldrich	D9779
Phosphatase inhibitor cocktail 2	Sigma-Aldrich	P5726
HEPES	Sigma-Aldrich	H3375
TEMED	Sigma-Aldrich	T9281
Ponceau S	Sigma-Aldrich	78376
Potassium hydroxide	Sigma-Aldrich	P5958
EDTA	Sigma-Aldrich	E5134
Glycerol	Sigma-Aldrich	G8773
Bromophenol blue	Sigma-Aldrich	B8026
Chymostatin	Sigma-Aldrich	C7268
Antipain dihydrochloride	Sigma-Aldrich	A6191
Leupeptain hemisulfate salt	Sigma-Aldrich	L2884
Pepstatin A	Sigma-Aldrich	P5318
Acrylamide	Sigma-Aldrich	A3699
Tween 20	Sigma-Aldrich	P1379
Ammonium persulfate	Sigma-Aldrich	A3678
Immobilon Western HRP substrate	Sigma-Aldrich	WBKLS0
Immobilon-P PVDF membrane	Sigma-Aldrich	IPVH00010
Whatman 3mm chromatography paper	Sigma-Aldrich	Z270857
Prestained protein marker, broad range	Sigma-Aldrich	P7708

A3 - List of antibodies

Primary Antibodies

Antibody	Supplier	Product code
β-actin	Cell Signalling	A1978 (clone AC-15)
Caspase-3	Cell Signalling	9662s
Caspase-9	Cell Signalling	9502s
JNK	Cell Signalling	9258s
Phospho-JNK	Cell Signalling	4668s
Lamin-B	Santa Cruz	sc-6217
MCL-1	Cell Signalling	6315s
MDR1	Sigma	P7965
P38	Cell Signalling	9212s
Phospho-p38	Cell Signalling	9216s

Secondary antibodies

Antibody	Supplier	Product code
Goat anti-mouse	Thermo Scientific	31430
Goat anti-rat	Thermo Scientific	31470
Goat anti-rabbit	Thermo Scientific	31460

A4 - List of buffers required for western- and immuno-blotting

10X PBS, pH 7.4

1.37M NaCl
2.7M KCl
1M Na_2HPO_4
20mM KH_2PO_4

10X TBS, pH 7.4

2.5M Tris
1.5M NaCl
200mM KCl

5X Laemmli sample buffer

10% SDS
50% Glycerol
300mM Tris, pH 6.8
0.1% Bromophenol Blue

PBST

1x PBS
0.05% Tween 20 [v/v]

TBST

1x TBS
0.05% Tween 20 [v/v]

1X Laemmli sample buffer

2% SDS
10% Glycerol
60mM Tris, pH 6.8
0.02% Bromophenol Blue
50mM DTT

10X Running Buffer

250mM Tris base
1.92mM Glycine
0.1% SDS

10X Transfer Buffer

2.5M Tris base
1.92M Glycine
10% Methanol (added fresh)

Ponceau S solution

0.1% Ponceau S
5% Acetic acid

Lysis Buffer

50mM HEPES-KOH, pH7.2
5mM EGTA
10mM KCl
2mM $MgCl_2$
2mM DTT*
1µl/ml CLAP*
1:100 Phosphatase Inhibitor I*
1:100 Phosphatase Inhibitor II**

Notes:
[*]: DTT and CLAP added fresh before use
[**]: Added when testing for phosphorylated proteins

A-5 List and status of cell lines used in this thesis

Cell Line	Tissue	TNBC	AR sensitive	ER+ve	PR+ve	HER2+ve	p53	PTEN
MDA-MB-468	Breast	Yes	N/A	No	No	No	MT	MT
MDA-MB-231	Breast	Yes	N/A	No	No	No	MT	WT
MDA16	Breast	Yes	N/A	No	No	No	MT	MT
Hs578t	Breast	Yes	N/A	No	No	No	MT	WT
HCC1937	Breast	Yes	N/A	No	No	No	MT	Null
BT20	Breast	Yes	N/A	No	No	No	MT	MT
MCF-7	Breast	No	N/A	Yes	No	Yes	MT	WT
T47D	Breast	No	N/A	No	Yes	No	WT	WT
SkBr3	Breast	No	N/A	No	Yes	No	MT	WT
BT-474	Breast	No	N/A	Yes	Yes	Yes	MT	WT
SW480	Colorectal	N/A	N/A	N/A	N/A	N/A	MT	Null
RKO	Colorectal	N/A	N/A	N/A	N/A	N/A	WT	MT
HT-29	Colorectal	N/A	N/A	N/A	N/A	N/A	MT	WT
HCT-8	Colorectal	N/A	N/A	N/A	N/A	N/A	WT	WT
DU145	Prostate	N/A	No	N/A	N/A	N/A	MT	MT
22Rv1	Prostate	N/A	No	N/A	N/A	N/A	WT	WT
PC-3	Prostate	N/A	No	N/A	N/A	N/A	MT	Null
MCF10A	Non-cancer breast	No	No	Normal	Normal	Normal	WT	WT

A6 Buffers and reagents required for TEM

Chemicals and Reagents:

Sodium cacodylate buffer **0.4M** (stock solution) - 21.4g sodium cacodylate dissolved in 250mL H_20. Store at 4°C

Sodium cacodylate buffer **0.2M** (working solution) - 50mL 0.4M sodium cacodylate stock + 45mL H_20. Add 1mL HCL to adjust pH to 7.2. Bring up to 100mL with H_20.

Sodium cacodylate buffer **0.1M** (50:50 0.2M: H_20).

Glutaraldehyde 25% EM grade. Store at 4°C.

Glutaraldehyde 3% in 0.2M sodium cacodylate buffer. Prepare just before using as follows:

6mL 25% gluteraldehyde
25mL 0.2M sodium cacodylate
Dilute to 45mL with H_20 and adjust to pH 7.3-7.4 with 1M HCL
Bring to 50mL with H_20

2% Osmium tetroxide **(HAZARD)** - 1 glass vial + 12.5mL H_20 in a dark **glass** bottle. Allow to dissolve for one hour.

1% Osmium tetroxide - 3mL osmium + 3mL H_20

30% Ethanol
50% Ethanol
70% Ethanol
95% Ethanol
100% Ethanol

50:50 resin:alcohol

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