THE EFFECT OF INHIBITING ER STRESS ON RESPONSE TO PACLITAXEL IN OVARIAN AND COLORECTAL CANCER CELLS

By

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ABSTRACT

The endoplasmic reticulum (ER) is the organelle responsible for carrying out the vital function of protein synthesis and secretion. Cellular disturbances cause the accumulation of unfolded proteins and induce a condition known as ER stress. This activates an evolutionary conserved multi-signalling pathway called the unfolded protein response (UPR) which tries to restore the cell homeostasis. However, prolonged ER stress causes the UPR to activate its apoptotic pathways to cause cell death. Studies have shown that ER stress and UPR induction influence the development of cancer through either its adaptive or apoptotic arms. In addition, it has been evidenced that UPR activation can sensitise cancer cells to certain chemotherapy drugs to cause either chemo-efficiency or chemo-resistance. The chemotherapy drug, paclitaxel, has been shown to use ER stress induced apoptosis to cause cell death. However, it is not exactly clear what mechanism paclitaxel uses to induce ER stress-mediated apoptosis. This research investigated paclitaxel response in ovarian and colorectal cancer cell lines by inhibiting known pathways of ER stress. From the results obtained, it was found that paclitaxel causes cellular death, but whether it does via the specific induction of an ER stress pathway is still not evident and needs further investigation.

DEDICATION

Dedicated to my family for all of their love and support. Thank you for always being all the good in me.

And to Adam. Whatever measure of a true scientist I am, I am because of your example, teachings, and guidance.

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LIST OF ABBREVIATIONS

APAF1	apoptotic protease activating factor-1
ATF4	activating transcription factor-4
ATF6	activating transcription factor-6
BAK	BCL-2 homologous antagonist/killer
BCL2	B-cell lymphoma-2
BiP	binding immunoglobulin protein
BSA	bovine serum albumin
cDNA	Complementary DNA
CHOP	C/EBPα -homologous protein
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
eIF2α	eukaryotic initiating factor 2α
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
FCS	foetal calf serum
FRA16D	common fragile site located on human chromosome 16q23
GRP78	78 kDa glucose-regulated protein
HSP60	60 kDa heat shock protein
IRE1	inositol requiring enzyme-1
JNK	c-Jun N-terminal kinase
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS/T	phosphate buffered saline with Tween

PDI	protein disulfide isomerase
PERK	protein kinase RNA-like ER kinase
RNA	Ribonucleic acid
RPM	revolutions per minute
RT PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SRB	sulforhodamine B
TCA	trichloroacetic acid
TEMED	Tetramethylethylenediamine
TRAF	TNF receptor-associated factor-2
TSG	tumour suppressor gene
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
WB	Western Blotting
WWOX	WW domain-containing oxidoreductase
XBP1	X-box binding protein-1

CHAPTER 1: INTRODUCTION

1.1 Cancer: an overview

Cancer is a group of diseases in which cells divide uncontrollably. Cancer cells arise from the accumulation of mutations within the DNA due to many environmental, and sometimes, genetic factors. These changes in the DNA turn normal healthy cells into abnormal ones that have unregulated growth. As these abnormal cells continue to proliferate, they form tumours which may be benign and self-limiting in their growth, or they may become malignant and spread to surrounding tissues, eventually metastasizing, or spreading to other parts of the body via the circulatory system. Cancer can develop in nearly any type of tissue, or part of the body, and most are typically named based on the tissue type or organ in which they originate. Currently, there are more than 200 types of cancer; lung, breast, colorectal, and stomach cancers being the most common. In the UK, breast, lung, colorectal and prostate cancers account for more than 50% of all cancer cases. Cancer is a leading cause of disease and death worldwide with an estimated 12.7 million people being diagnosed with cancer in 2008, and 7.6 million dying from it the same year. If current trends in leading cancer types continue, there will be an estimated 22 million new cases each year by 2030 (Cancer Research UK and IARC, 2011).

1.1.1 Ovarian cancer

Ovarian cancer is a tumour growth occurring in the ovary. This is due to malignant change of the surface of the ovary known as the epithelium. The symptoms of ovarian cancer are nonspecific as they are often similar to that of upper abdominal disease such as: fullness, indigestion, and bloating. Some early stage patients have pelvic pain though most patients are asymptomatic in the early stage of the disease (Cannistra, 2004). In the UK, ovarian cancer is the fifth most common cancer and the fourth most common cause of cancer related death in women with 4,400 deaths in 2008. This accounts for more deaths than all the other gynaecological cancers combined (Cancer Research UK, 2011). There are approximately 6,600 new cases diagnosed each year in the UK (Paige and Brown, 2008) with 1 in 50 UK women having the risk of developing ovarian cancer (Cancer Research UK, 2011). Normal treatment for ovarian cancer is surgical removal of the tumour followed by chemotherapy consisting of a platinum-containing drug (e.g. carboplatin) and a taxane (e.g. paclitaxel). Ovarian cancer is considered a chemo-sensitive tumour since over 90% of patients respond to first line chemotherapy. However, three quarters of patients will relapse within a few years

with chemo-resistance occurring in the majority of cases, resulting in poor survival rates as low as 42.9% after 5 years (Paige and Brown, 2008).

1.1.2 Colorectal Cancer

Colorectal cancer, or bowel cancer, is the uncontrolled growth of cells in the colon and/or the rectum. The malignant change usually occurs in the gland cells making up the mucosal lining of the intestinal walls. The symptoms of colorectal cancer most commonly include bleeding of the rectum, change in normal bowel movements, and weight loss. In the UK, colorectal cancer is the fourth most common cancer and the second most common cause of cancer related death. There are approximately 40,000 new cases diagnosed each year with more than 80% of cases occurring in people aged 60 or older. Normal treatment includes surgical removal of the infected areas followed by chemotherapy and radiotherapy. More than 90% of patients survive 5 years or more if diagnosed in the early stages (Cancer Research UK, 2013).

1.2 WWOX: a bona fide tumour suppressor gene

The WW domain–containing oxidoreductase (WWOX) gene is over 1 Mb in size and is located on chromosome 16 at the region, FRA16D, a common fragile site involved in cancer. The WWOX gene is composed of nine exons and encodes a 46 kDa protein having two *N*-terminal WW domains and an oxidoreductase domain (Gourley et al., 2009; Hezova et al., 2007). WWOX has been shown to have multiple functions such as: interacting with proline-containing ligands, mediating protein-protein interactions, metabolising steroid hormones, and its up-regulation in endocrine organs such as testis, ovary and breast, indicate its importance in these tissues (Hezova et al., 2007). The homozygous deletion of WWOX has been observed in a number of cancers, and decreased or abnormal expression of WWOX has been shown in multiple tumour types (Paige et al., 2001). In addition, several studies have shown that the targeted deletion of WWOX in mice causes increased tumorigenicity confirming that WWOX is a bona fide tumour suppressor gene (TSG) (Gourley et al., 2009). The WWOX tumour suppression mechanism has been linked to the induction of apoptosis. The induction of apoptosis via a caspase dependent mechanism was observed after WWOX transfection in lung and prostate tumours (Qin et al., 2006). WWOX has been shown to directly bind to p73 protein, a structural and functional homologue of the p53 tumour suppressor protein, causing enhanced p73-mediated apoptosis (Aqelian et al., 2004). It has also been reported that WWOX can bind the p53 tumour suppressor protein and enhance the apoptotic response to tumour necrosis factor (Chang et al., 2009). However, other possible

mechanisms of WWOX tumour suppression have been suggested since studies in breast cancer showed suppression of tumorigenicity was not associated with increased apoptosis (Bedmarek et al., 2001; Hezova et al., 2007).

1.2.1 The role of WWOX in ovarian cancer

It has been previously identified that WWOX is disrupted by homozygous deletion and shows frequent decreased expression in ovarian cancer (Gourley et al. 2009). In addition, a study by Nunez and colleagues showed, using a panel of 444 human ovarian cancers, that 30% of tumours had significantly low levels of WWOX expression, and that this was even more common in the two most aggressive ovarian cancer types, with 70% in mucinous and 42% in clear cell carcinomas. This loss of WWOX expression in ovarian tumorigenesis may result from methylation of the WWOX promoter region (Iliopoulos et al., 2005; Hezova et al., 2007). It has been evidenced that WWOX transfection of human PEO1 ovarian cancer cells, containing homozygous WWOX deletion, abolishes in vivo tumorigenicity (Gourley et al., 2009). In addition, it has been demonstrated in the research group of Dr Adam Paige that WWOX reconstitution in ovarian cancer cells inhibited xenograft growth, but that this did not alter *in vitro* growth or apoptosis rates. Instead, transfection of WWOX reduced cancer cell adhesion to ECM (extracellular matrix) by reducing integrin binding (Janczar, unpublished). Based on the hypotheses that WWOX regulated gene expression or promoted apoptosis in ovarian cancer, Dr Szymon Janczar, a former PhD of Dr Paige, showed in his previous studies that WWOX increased apoptosis rates in ovarian cancer cells treated with the chemotherapy drug paclitaxel, and that this was independent of mitotic arrest mechanism of taxanes and unrelated to integrin regulation. Instead, he demonstrated that WWOX promoted cell death during paclitaxel-induced endoplasmic reticulum stress. He proposed that WWOX-induced cell death during endoplasmic reticulum stress may be linked to anti-tumorigenic effects in vivo (Janczar, unpublished). Based on this previous work, this research proposed to further investigate the endoplasmic reticulum stress-induced apoptosis in paclitaxel response.

1.3 The endoplasmic reticulum and ER stress

The endoplasmic reticulum (ER) is a specialized organelle responsible for carrying out cellular functions such as: protein folding, lipid biosynthesis, and calcium and redox homeostasis; all of which play crucial roles in cell homeostasis and survival (Ozcan and Tabas, 2012). The lumen of the ER acts as the major site for the production and transport of proteins, and uses a process known as the ER associated degradation (ERAD) to monitor the

quality of all the proteins assembled within in it (Tsai and Weissman, 2010). Only proteins which have been folded properly are allowed to be transported out, while improperly folded, or unfolded, proteins are kept to complete the folding process or taken to the cytosol to be degraded (Ozcan and Tabas, 2012). The lumen is a uniquely oxidative environment essential for the formation of disulfide bonds needed in the proper folding of proteins, and is highly concentrated with calcium and calcium dependent chaperones, such as GRP78, GRP94, protein disulphide isomerase (PDI), calnexin, and calreticulin. All of these chaperones help with the proper folding and maturation of proteins as important parts of the ERAD (Xu et al., 2005). In addition to protein folding, the ER is also involved in lipid biosynthesis and monitors the level of oxygen and nutrients in cells as they adapt to their environment (Tsai and Weissman, 2010). All of these functions create a very dynamic environment within the ER. Making the biosynthesis occurring within the ER far greater than that which takes place in the cytosol (Ma and Hendershot, 2004). Under normal physiological conditions, the ER keeps a balance between its protein load and protein folding capacity, and helps to establish ER homeostasis. However, changes in its homeostasis due to increased levels of protein, or accumulation of unfolded proteins causes a condition known as ER stress (Ozcan and Tabas, 2012).

1.4 ER stress and the unfolded protein response

Physiological disturbances like hypoxia, glucose deprivation, oxidative stress, viral infection, high cholesterol levels, and genetic mutations change the ER homeostasis and cause the accumulation of unfolded proteins which in turn leads to ER stress (Tsai and Weissman, 2010). To cope with this stress, cells activate an evolutionary conserved signalling pathway called the unfolded protein response (UPR). The primary objective of the UPR is to re-establish homeostasis. It accomplishes this in three ways: (1) increasing the protein folding capacity of the ER via expression of protein folding chaperones, (2) the degrading of unfolded proteins via enhancement of components of the ERAD, and (3) reducing the ER protein load via inhibition of protein translation. However, if ER stress is prolonged or severe, the secondary objective of the UPR is to trigger programmed cell death, or apoptosis (Ozcan and Tabas, 2012; Tsai and Weissman, 2010; Xu et al., 2005).

1.4.1 Mechanism of the UPR

The UPR has three major pathways, each dependent on the activation of an ER localized transmembrane molecule: PERK (protein kinase RNA-like ER kinase); IRE1 (inositol

requiring enzyme-1); and ATF6 (activating transcription factor-6). These three transmembrane molecules are thought to be kept in an inactive state by an interaction with binding immunoglobulin protein (BiP), also known as the ER chaperone, GRP78 (78 kDa glucose-regulated protein). As unfolded proteins begin to accumulate in the lumen of the ER, BiP releases the three transmembrane molecules in order to bind to the unfolded proteins, and in turn, allowing the activation of the molecules. However, it has been recently suggested that there may be other means by which activation occurs such as direct binding of unfolded protein to the transmembrane molecules (Ozcan and Tabas, 2012). Figure 1 illustrates the UPR and the activation of its three pathways. Activated PERK, a serine threonine kinase, phosphorylates eukaryotic initiation factor 2a (eIF2α) which stops mRNA translation, lowering the protein load in the ER. However, the selective translation of certain proteins, such as activating transcription factor 4 (ATF4), which is responsible for the regulation of genes required in UPR, is allowed through an alternate pathway (Duffee et al., 2012; Li et al., 2011; Xu et al., 2005). The transmembrane molecule, ATF6, a leucine zipper transcription factor, translocates to the Golgi apparatus and is cleaved by two proteases. The cleaved ATF6 moves to the nucleus and induces the expression of ER chaperones like GRP78 (Ozcan and Tabas, 2012). IRE1, the most evolutionary conserved of the three, transautophosphorylates and works in conjunction with ATF6 to produce the X-box binding protein-1 (XBP1). First, ATF6 begins the transcription of an incompletely spliced, immature form of XBP1 mRNA. Then the cytoplasmic endoribonuclease domain of IRE1 splices the mRNA into a mature form, creating the proper open reading frame, which can be translated into XBP1 protein (Duffee et al., 2012; Li et al., 2011; Xu et al., 2005). XPB1 is a transcription factor essential for hepatocyte growth, the differentiation of plasma cells, immunoglobulin secretion, and inducing many UPR genes like: GRP78, BiP, EDEM, RAMP-4, PDI-P5, and HEDJ, all of which appear to act in the ER and increase its folding capacity and secrete unfolded proteins via the ERAD (Lee et al., 2003; Yoshida et al., 2001). Because the splicing of the XBP1 mRNA by IRE1 occurs in the cytoplasm instead of the nucleus, it is considered to be unconventional (Uemura et al., 2009).

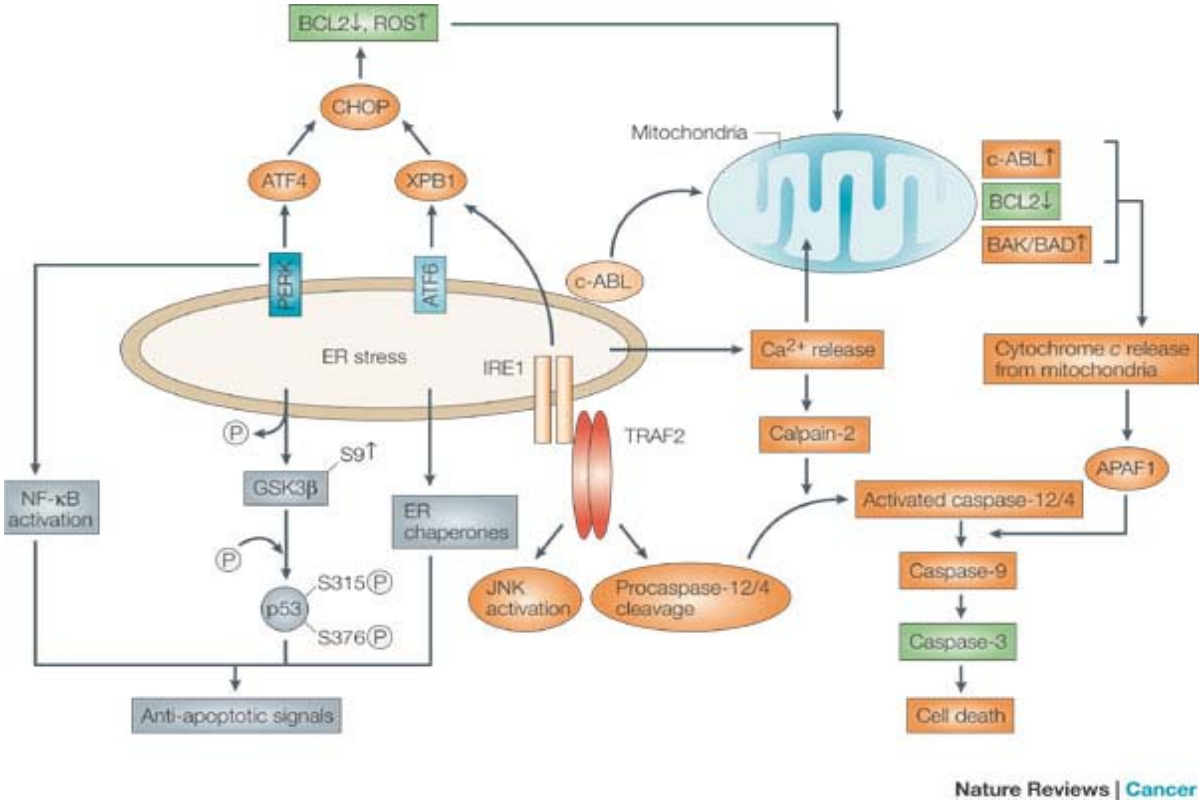


FIGURE 1: The pro- and anti-apoptotic roles of the unfolded protein response pathway
(Image taken from Ma and Hendershot, 2004).

1.4.2 The apoptotic arm of the UPR

As stated previously, the initial objective of the UPR is to re-establish homeostasis, but prolonged or severe ER stress can cause the UPR to activate its apoptotic mechanisms. As illustrated in Figure 1, all three UPR pathways have shown to have pro-apoptotic proteins downstream of their activation. For example, upon activation, IRE1 binds TRAF2 (TNF receptor–associated factor-2) which promotes activation of JNK (c-Jun N-terminal kinase). In addition, IRE1 activation has shown to trigger the recruitment of caspase-12 or caspase-4, a pro-apoptotic cysteine protease. In the PERK pathway, phosphorylated eIF2α initiates translation of ATF4 (activating transcription factor-4) which in turn induces the UPR effector CHOP (C/EBPα-homologous protein). The prolonged expression of CHOP causes down regulation of the anti-apoptotic agent BCL2 (B-cell lymphoma-2) and up regulates pro-apoptotic agent BAK (BCL-2 homologous antagonist/killer) (Ozcan and Tabas, 2012). This causes mitochondrial membrane damage and releases cytochrome c which then activates APAF1 (apoptotic protease activating factor-1). APAF1 triggers caspase-9 activation which in turn activates caspase-3 causing cell death. The possible involvement of a mitochondria-independent pathway in ER stress induced apoptosis has also been proposed. In this pathway,

the increase of calcium levels caused by calcium release from the ER to the cytosol during ER stress activates calpain which induces cleavage of procaspase-12 into caspase-12. Caspase-12 then activates caspase-9, promoting cell death (Li et al., 2011). The exact point which determines this switch from adaptive response to apoptotic response is not clearly understood since the activation times for the three UPR pathways differ, with IRE1 activity being quickly attenuated, while PERK and ATF6 activity is more prolonged. But, it has been suggested that the different activation times do play a role in the switch (Ma and Hendershot, 2004; Tsai and Weissman, 2010).

1.5 The UPR and cancer development

As previously mentioned, changes to the ER brought about by physiological disturbances such as hypoxia, glucose deprivation, oxidative stress and genetic mutations cause the accumulation of unfolded proteins leading to ER stress. Cancer cells contribute many of these disturbances due to their increased rate of growth. Since cancer cells need a much higher level of protein production, and also express mutant proteins that cannot be folded properly, they add pressure and tip the delicate balance of the ER. As cancer cells rapidly proliferate and begin to metastasize, they start to have poor vascularization, experience hypoxia and nutrient starvation, all of which lead to ER stress and the activation of the UPR. It has been evidenced in several studies that the induction of ER stress and the consequent activation of the UPR play a prominent role in cancer development (Mann and Hendershot, 2006) and that certain products of the UPR pathway contribute in enhancing the life of cancer cells. For example, cancer cells produce promoters of angiogenesis to form new blood vessels in order to receive nutrients. VEGF (vascular endothelial growth factor), a pro-angiogenic protein often found in tumours, is up regulated during ER stress via the PERK pathway and its secretion is regulated by GRP170, an ER stress activated chaperone (Ma and Hendershot, 2004). So, as cancer cells proliferate causing unfolded proteins to accumulate and lead to ER stress, the responsive products of the UPR increase the activation of growth factors needed for the cancer cells to grow and spread, forming a vicious cycle. Due to this, some cancers have actually evolved to have ER stress inherently activated with in them and use certain UPR signals to bypass even its own apoptotic pathways. Many of these ER stress inherited cancers display altered expression patterns of ER-resident proteins. Two such proteins are the ER chaperones GRP78 and GRP94 whose expression is increased in at least 10 different cancers including lung, breast, and colon cancers. Both chaperones have been shown to play a role in the ability of cancer cells to avoid pro-apoptotic pathways (Moenner et al., 2007).

Whilst some cancers likely use UPR to promote cell survival, in other tumours where UPR is not inherently activated it may play a role in killing cancer cells via its apoptotic arm. Data from cell culture studies showed that pharmacological activation of the UPR changes the chemotherapeutic sensitivity of cancer cells; sensitizing them to some drugs while making them resistant to others (Mann and Hendershot, 2006). Considering all this, inoculation of cancer cells with either ER stress inducing or inhibiting agents before chemotherapy drug treatments may aid in the apoptotic response of the drug.

1.6 ER stress and chemotherapy

As stated above, it has been evidenced that therapeutic induction of ER stress induced apoptosis may be helpful in killing cancer cells as the activation of the UPR sensitizes them to certain chemotherapy drugs. One such chemotherapy drug is cisplatin, a platinum based drug, which works by binding to DNA and creating cross links which prevent cells from dividing by mitosis. Cisplatin is used to treat various types of cancer including ovarian, colorectal, lung and testicular cancers. In-vitro treatment of cancer cells have shown that ER stress sensitizes cancer cells to cisplatin, and causes apoptotic death via a calpain-dependent activation of caspase-12. This shows that inducing ER stress in cancer cells prior to chemotherapy could help in the activation of an apoptotic pathway similar to that seen in cisplatin response (Ma and Hendershot, 2004).

1.6.1 Paclitaxel

Another drug which has shown to be affected by the activation of the UPR is paclitaxel. Paclitaxel is a chemotherapy drug isolated from the bark of the yew tree and is used as the first line treatment for ovarian, colorectal, breast and lung cancer. Paclitaxel is classified as a mitotic inhibitor because it binds to tubulin to promote microtubule assembly which interferes with the normal breakdown of microtubules resulting in disruption of mitosis at the G2/M phase (Ofir et al., 2001). Though this is its primary apoptotic mechanism, it has been suggested that paclitaxel may also use an ER stress activated mechanism to cause cell death. Studies conducted by Dr Szymon Janczar demonstrated that cell death due to paclitaxel exposure is mediated by ER stress, and that paclitaxel evoked ER stress in PEO1 cells, an ovarian cancer line (Janczar, unpublished). In addition, studies using thapsigargin, a classic ER stress inducer, showed to increase the cell killing potency of paclitaxel by tenfold (Wu et al., 2009). It may be very beneficial then to test paclitaxel response in conjunction with drugs that either induce or inhibit ER stress in other cancer cell lines.

1.7 Aims

The induction of ER stress and the activation of the UPR seem to influence the development of cancer by either favouring an adaptive or apoptotic response. It can be argued, whether the activation of the pathway is more beneficial for the host or the cancer in every case as current data seems to evidence either. Despite the opposing arguments, it is clear that future applications will depend on studies to test which specific chemotherapeutic drugs are sensitized by ER stress and the UPR pathway. Understanding the nature of the apoptotic mechanisms of ER stress may help in not only overcoming chemotherapy drug resistance in specific cancers, but also, to further improve chemotherapeutic efficacy for individual cancer patients. In light of the evidence and data collected from previous studies, this research investigated the effect of inhibiting ER stress in response to paclitaxel in ovarian and colorectal cancer cells. The aims of this research were as follows:

1. Target ER stress in order to inhibit paclitaxel chemotherapy response
2. Characterize the activation of ER stress by paclitaxel in different cancer cell lines

1.7.1 Targeting ER stress in order to inhibit paclitaxel chemotherapy response

This was accomplished by targeting and inhibiting the ER stress associated enzymes: caspase-4, caspase-12, and JNK. In addition, the experiments used salubrinal to observe whether its use reversed paclitaxel function by inhibiting ER stress. Cell survival following the drug treatments was determined by SRB staining the cells and measuring the level of absorbance via spectrophotometric analysis. Since paclitaxel is also used as a first line treatment for colon cancer, the colorectal cancer cell lines, HCT116 and HCT116 Bax null were used for the experiments in addition to the ovarian cancer cell lines, A2780 and SKOV3. Use of the HCT null variant line allowed us to investigate the key role of the intrinsic apoptosis protein BAX in the UPR apoptotic pathway used in paclitaxel response.

1.7.2 Characterizing the activation of ER stress by paclitaxel in different cancer cell lines

Experiments were run to observe the effects of the chemotherapy drug, paclitaxel, on various cancer cell lines in order to see if application of the drug induced ER stress, and whether this activation led to apoptosis. Cell survival following the drug treatments were determined by staining the cells with sulforhodamine B (SRB), a protein coating dye, and measuring the

level of absorbance via spectrophotometric analysis. The protein detecting technique of western blotting was used to look at the level of proteins involved as part of the ER stress pathway. These experiments were aimed to identify in which cancer cell lines ER stress was inherently activated, whether there was induction of ER stress by paclitaxel, and whether the various inhibitors blocked the unfolded protein response.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture

All cell lines (including those used for experiments) were observed and monitored under the microscope during their growth and the duration of the experiments.

2.1.1 Basic maintenance of cell lines

All cell culture work was conducted in a sterile environment of a class II laminar flow safety cabinet. Established ovarian and colorectal cancer cell lines were kept in RPMI-1640 medium with HEPES (PAA #E15-041) supplemented with 10% FBS (foetal bovine serum) (PAA #A15-152), 0.4 mM L-glutamine (PAA #M11-004), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA #P11-010), 1% amphotericin B (Sigma #A2942) in an atmosphere of 5% CO_2 at 37°C. The ovarian cell lines: A2780 and SKOV3, and the colorectal cell lines: HCT116 and HCT116 Bax null were used.

2.1.2 Passaging of cell lines

The adherent cell lines were grown in 75 cm^2 flasks until they reached 90-100% confluence (cell line dependent). The cells were washed with PBS (phosphate buffered saline) (Oxoid #BR0014) and then detached from the flask using trypsin (Sigma #T4549) before being split into new flasks containing RPMI medium and placed in an atmosphere of 5% CO_2 at 37°C.

2.1.3 Freezing and recovery of cell lines

The cell lines were frozen down using a freeze mix of 90% FBS and 10% DMSO (dimethyl sulfoxide) (Sigma #D4540) after being washed with PBS and trypsinized as normal. The cells were kept in cryo-tubes stored in liquid nitrogen. Frozen cells were recovered in RPMI medium before being centrifuged at 1,200 rpm for 5 minutes. The supernatant was discarded, and the cells were re-suspended in RPMI medium and transferred into 25 cm^2 flasks which were placed in an atmosphere of 5% CO_2 at 37°C.

2.2 Cell survival assays

All cell survival experiments were repeated multiple times, independently of each other for each cell line.

The statistical significance of intergroup differences was determined using Student's t-test. P values ≤ 0.05 were considered statistically significant.

2.2.1 ER stress inhibition and cell survival in HCT116 cell line

2,000 log-phase HCT116 cells/200 µl per well (in triplicate for each drug treatment) were plated into flat bottom 96-well plates. After 48 hours, the medium was removed and replaced with either only medium, or medium containing 32 nM paclitaxel (#), 30 µM salubrinal (Merck #324895), 60 µM salubrinal, 32 nM paclitaxel with 30 µM salubrinal, 32 nM paclitaxel with 60 µM salubrinal, 200 ng/ml tunicamycin (Sigma #T7765), 200 ng/ml tunicamycin with 30 µM salubrinal, 200 ng/ml tunicamycin with 60 µM salubrinal, or 0.02% DMSO. After a further 72 hours, the cells were fixed with 10% TCA (trichloroacetic acid) (Sigma #T6399) and incubated for 1 hour at 4°C. The plates were then washed with cold tap-water, dried and stained with 0.4% SRB (sulforhodamine B) (Sigma #230162) solution, washed with 1% acetic acid (Sigma #320099) and dried again. The dye was re-solubilized with 10 mM Tris (Sigma #93362) and the absorbance was read at 492 nm wavelength.

2.2.2 ER stress inhibition and cell survival in HCT116 Bax null cell line

4,000 log-phase HCT116 Bax null cells/200 µl per well (in triplicate for each drug treatment) were plated into flat bottom 96-well plates. After 48 hours, the medium was removed and replaced with either only medium, or medium containing 32 nM paclitaxel, 30 µM salubrinal, 60 µM salubrinal, 32 nM paclitaxel with 30 µM salubrinal, 32 nM paclitaxel with 60 µM salubrinal, 200 ng/ml tunicamycin, 200 ng/ml tunicamycin with 30 µM salubrinal, 200 ng/ml tunicamycin with 60 µM salubrinal, or 0.02% DMSO. After a further 72 hours, the cells were fixed with 10% TCA and incubated for 1 hour at 4°C. The plates were then washed with cold tap-water, dried and stained with 0.4% SRB solution, washed with 1% acetic acid and dried again. The dye was re-solubilized with 10 mM Tris and the absorbance was read at 492 nm wavelength.

2.2.3 ER stress inhibition and cell survival in A2780 cell line

15,000 log-phase A2780 cells/200 µl per well (in triplicate for each drug treatment) were plated into flat bottom 96-well plates. After 48 hours, the medium was removed and replaced with either only medium, or medium containing 16 nM paclitaxel, 30 µM salubrinal, 200 ng/ml tunicamycin, 16 nM paclitaxel with 30 µM salubrinal, 30 µM salubrinal with 200 ng/ml tunicamycin, 10 µM caspase-4 inhibitor (Z-YVAD-FMK) (R&D #FMK005), 10 µM

caspase-4 inhibitor with 16 nM paclitaxel, 10 µM caspase-12 inhibitor (Z-ATAD-FMK) (R&D #FMK013), 10 µM caspase-12 inhibitor with 16 nM paclitaxel, 10 µM JNK inhibitor (SP600125) (Tocris Bioscience #1496), 10 µM JNK inhibitor with 16 nM paclitaxel, 0.05 % or 0.18% DMSO. After a further 72 hours, the cells were fixed with 10% TCA and incubated for 1 hour at 4°C. The plates were then washed with cold tap-water, dried and stained with 0.4% SRB solution, washed with 1% acetic acid and dried again. The dye was re-solubilized with 10 mM Tris and the absorbance was read at 570 nm wavelength.

2.2.4 ER stress inhibition and cell survival in SKOV3 cell lines

6,000 log-phase SKOV3 cells/200 µl per well (in triplicate for each drug treatment) were plated into flat bottom 96-well plates. After 48 hours, the medium was removed and replaced with either only medium, or medium containing 20 µM paclitaxel, 30 µM salubrinal, 200 ng/ml tunicamycin, 20 µM paclitaxel with 30 µM salubrinal, 30 µM salubrinal with 200 ng/ml tunicamycin, 10 µM caspase-4 inhibitor (Z-YVAD-FMK), 10 µM caspase-4 inhibitor with 20 µM paclitaxel, 10 µM caspase-12 inhibitor (Z-ATAD-FMK), 10 µM caspase-12 inhibitor with 20 µM paclitaxel, 10 µM JNK inhibitor (SP600125), 10 µM JNK inhibitor with 20 µM paclitaxel, 0.05 % or 0.18% DMSO. After a further 72 hours, the cells were fixed with 10% TCA and incubated for 1 hour at 4°C. The plates were then washed with cold tap-water, dried and stained with 0.4% SRB solution, washed with 1% acetic acid and dried again. The dye was re-solubilized with 10 mM Tris and the absorbance was read at 570 nm wavelength.

2.3 Protein analysis

2.3.1 Western blotting

Cells were washed with cold PBS and lysed using 1X Laemmli buffer (62.5 mM Tris HCl pH=6.8, 2% SDS, 10% glycerol) supplemented with 0.1M Protease Inhibitor Cocktail (Sigma #P8340) before being detached with a cell scraper and collected into Eppendorf tubes which were then centrifuged at 12,000 rpm for 5 minutes in 4°C. Total protein concentration in the lysates was quantified with BCA Protein Assay Kit (Pierce #23225) using the manufacturer's instructions. The lysates were supplemented with DTT (dithiothreitol) (Sigma #43816) to the final concentration of 1 M and boiled at 95°C for 5 minutes. 1X loading buffer (1X Laemmli buffer, 0.004% bromophenol blue, 1 M DTT) was added to the lysates before the proteins were separated on Mini-Protean TGX Precast Gels (10% resolving gel) (Bio Rad #456-1033)

using 1X running buffer (25 mM Trizma base, 192 mM glycine, 0.1% SDS) in a Mini-PROTEAN Tetra Cell (Bio Rad #165-8005TGX). The separated proteins were then transferred onto nitrocellulose membrane (Bio Rad #162-0115) using 1X transfer buffer (20% methanol, 0.37% SDS, 20 mM Trizma base, 154 mM glycine) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio Rad #170-3935TGX). The protein transfer was then verified by Ponceau S solution staining (Sigma #P7170). The membranes were blocked in 2% BSA (bovine serum albumin) (Sigma #A9647) in PBS/T (phosphate buffered saline with Tween) for 2 hours at room temperature, and then incubated with the appropriate dilution of the primary antibody (alkaline phosphatase conjugate) overnight at 4°C on a shaker. The membranes were washed several times with PBS/T and incubated with the appropriate dilution of the secondary antibody (alkaline phosphatase conjugate) for 1 hour at room temperature on a shaker. After several washes with PBS/T, the membranes were treated with SIGMAFAST BCIP/NBT tablets (alkaline phosphatase substrate) (Sigma #B5655) dissolved in distilled water to visualize protein bands. The membranes were incubated in the BCIP/NBT solution for 1-2 minutes at 4°C on a shaker.

The primary antibodies used for western blotting are characterized in Appendix 1.

2.3.2 Time course experiments using A2780 cell line

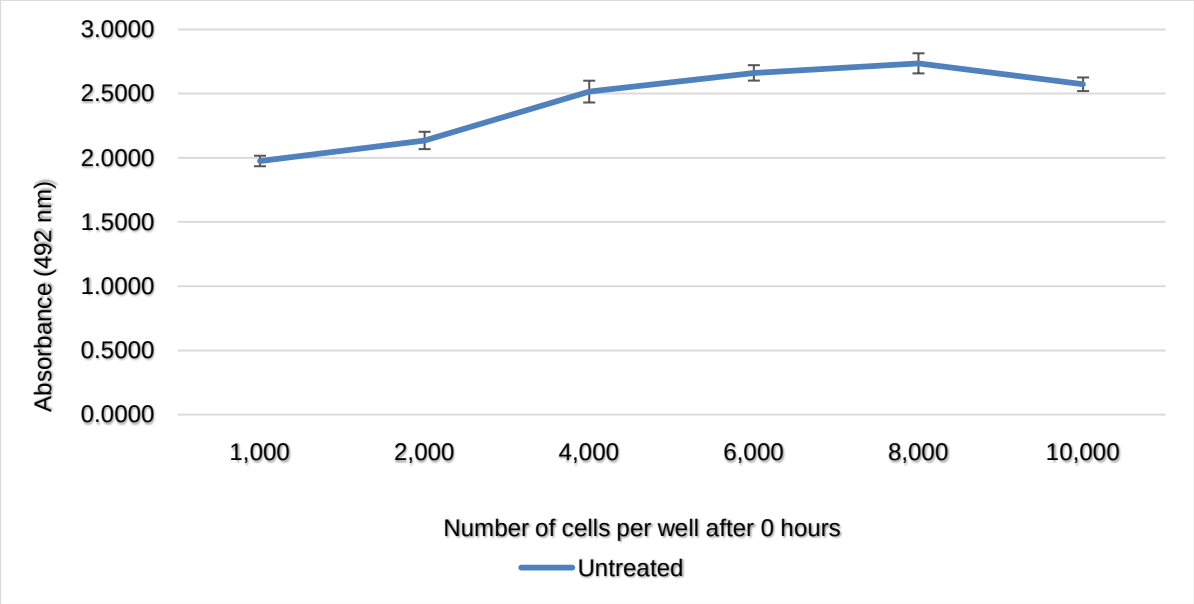
A2780 log-phase cells were counted in a haemocytometer and 6.5 x 10^4 cells per well (in duplicates for each drug treatment) of a 6-well plate were plated in 3 ml of RPMI medium. After 48 hours, the medium was removed and replaced with medium containing either 16 nM paclitaxel, 16 nM paclitaxel with 30 µM salubrinal, or 16 nM paclitaxel with 10 µM JNK inhibitor (SP600125). Following exposure times of either 0, 2, 4, 8, 12, 24, or 36 hours, the drug treatments were stopped by removing the medium. The cells were then lysed and prepped for protein analysis via the western blotting protocol described above in 2.3.1.

CHAPTER 3: RESULTS

3.1 Determining appropriate cell densities for HCT116 and HCT116 Bax null cell lines

Before starting the main investigative aims of this research, preliminary work was conducted to determine the appropriate cell densities and drug concentrations. First, an experiment to determine the appropriate cell densities for the colorectal cell lines: HCT116 and HCT116 Bax null was conducted. The two cell lines were plated at various cell densities (in triplicates) and grown for 120 hours. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each cell density was averaged to obtain the mean absorbance. The lowest cell density that reached near full confluence after 120 hours was chosen as the appropriate cell density. Figure 2 shows this to be at the cell density of 2,000 cells in the HCT116 cell line and at 4,000 for the HCT116 Bax null cell line. Having near full confluence instead of total confluence allowed for the optimum growing condition for the drug treatments by having the cells still in their log phase at the end of the experiment, ensuring that the cells had not yet reached the plateau phase and so any decrease in the cell density was purely in response to the drug treatments.

A **HCT116**



B **Bax null**

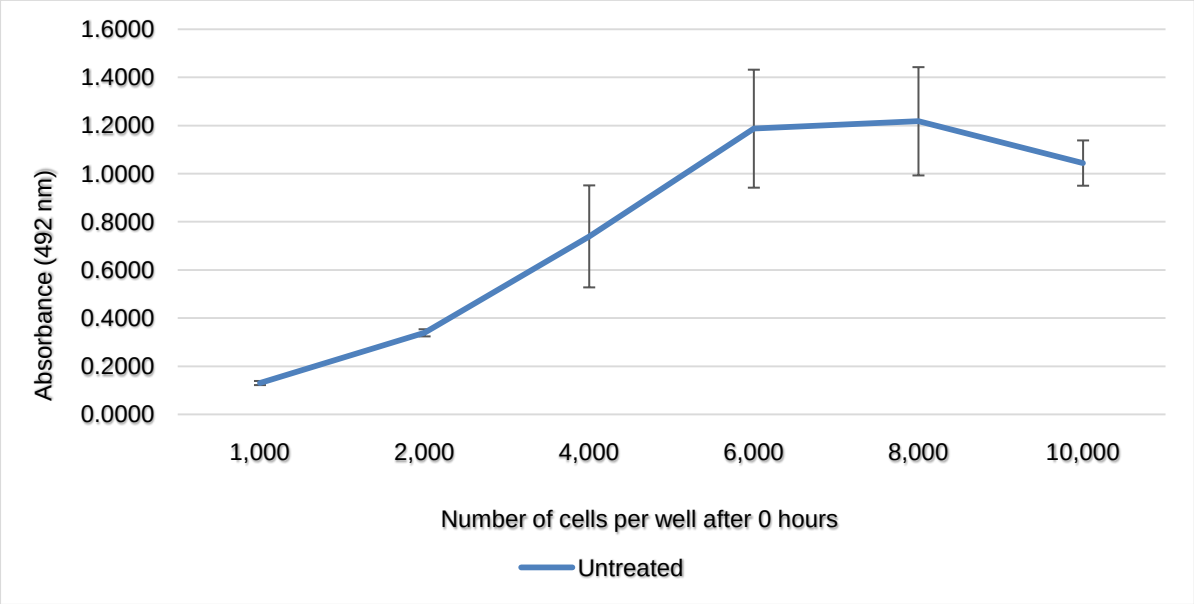
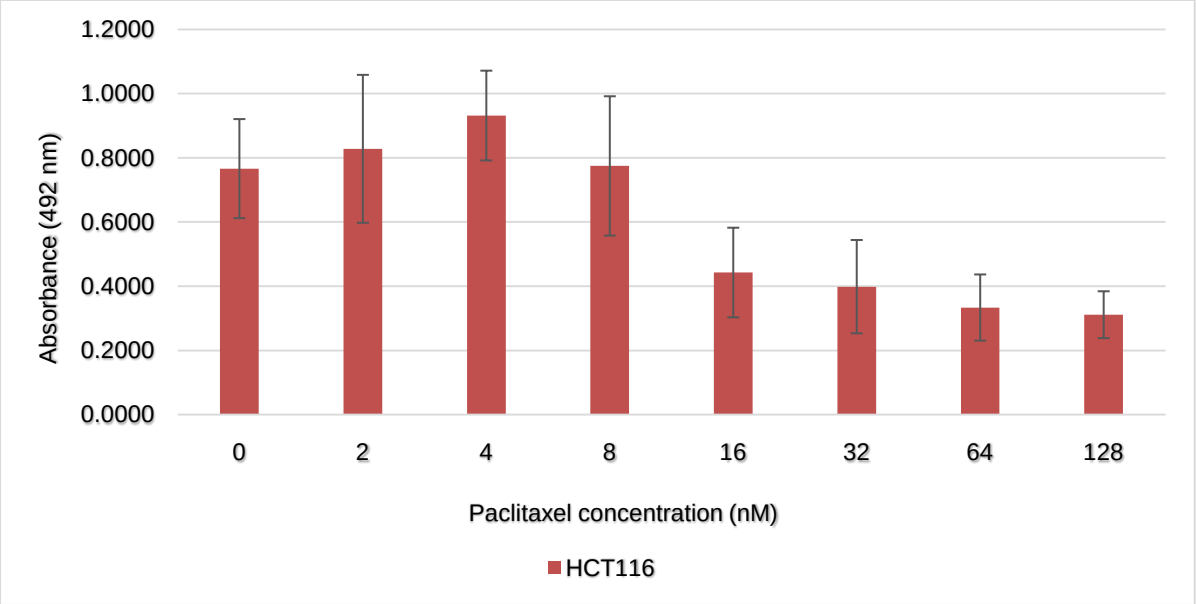


FIGURE 2: Cell densities of colorectal cancer cell lines after 120 hours
(A) HCT116 cells at varying plating densities. After 120 hours, spectrophotometric analysis was used (post SRB staining) to determine the lowest cell density that reached near full confluence. (B) HCT116 Bax null cells at varying plating densities. After 120 hours, spectrophotometric analysis was used (post SRB staining) to determine the lowest cell density that reached near full confluence. Data represents means of triplicate wells ± standard deviation.

3.2 Determining appropriate paclitaxel concentration to use on HCT116 and HCT116 Bax null cell lines

Next, an experiment to determine the appropriate paclitaxel concentration to use on HCT116 and HCT116 Bax null cell lines was conducted. Based on the cell density experiments, 2,000 HCT116 cells and 4,000 HCT116 Bax null cells were plated per well (in triplicates) and grown for 48 hours. Eight different concentrations of paclitaxel were used to treat the cells for another 72 hours. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each drug concentration was averaged to obtain the mean absorbance. The paclitaxel concentration at which approximately fifty percent of the cells survived was chosen as the appropriate drug concentration. Figure 3 shows this to be at the paclitaxel concentration of 32 nM for both the HCT116 and HCT116 Bax null cell lines. The absorbance readings for both cell lines at the 32 nM concentration is reduced nearly by half when compared to that of the untreated, indicating that only half the number of cells survived.

A **HCT116**



B **Bax null**

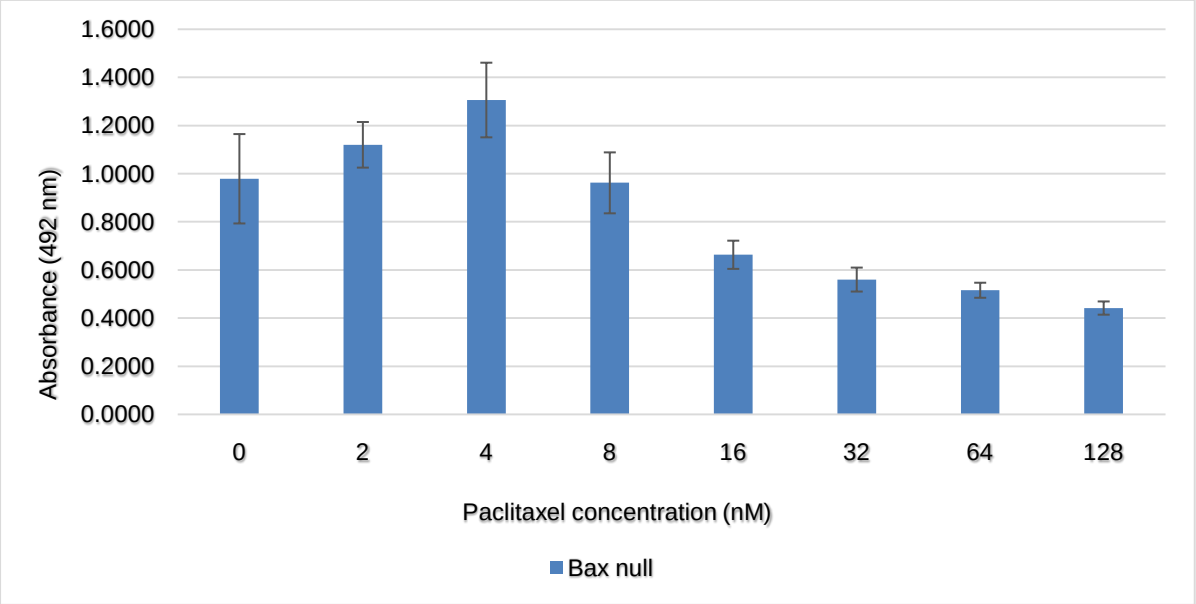
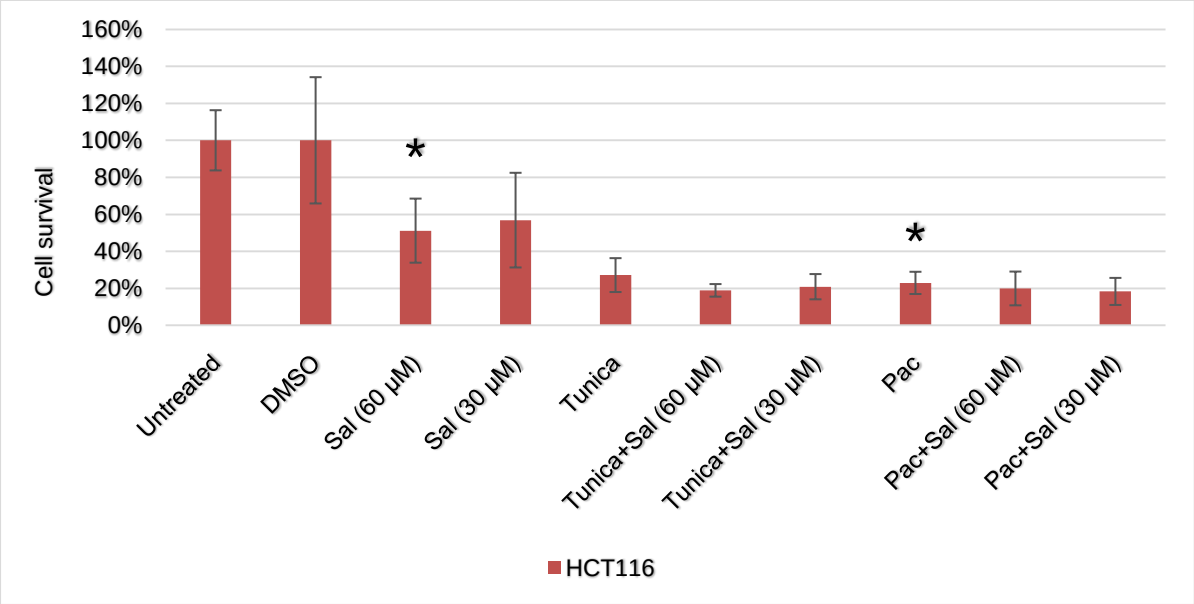


FIGURE 3: Cell densities of colorectal cancer cell lines at varying paclitaxel concentrations.
(A) HCT116 cells were treated with different paclitaxel concentrations (0, 2, 4, 8, 16, 32, 64, or 128 nM). After 72 hours, spectrophotometric analysis was used (post SRB staining) to determine the paclitaxel concentration at which approximately fifty percent of the cells survived. (B) HCT116 Bax null were cells treated with different paclitaxel concentrations (0, 2, 4, 8, 16, 32, 64, or 128 nM). After 72 hours, spectrophotometric analysis was used (post SRB staining) to determine the paclitaxel concentration at which approximately fifty percent of the cells survived. Bars represent means of triplicate wells ± standard deviation.

3.3 Paclitaxel treatment and ER stress inhibition in HCT116 and HCT116 Bax null cell lines

Previous studies have shown that paclitaxel induces cellular apoptosis via ER stress and the UPR pathways (Mhaidat et al., 2011). In this experiment, the colorectal cancer cell lines, HCT116 and HCT116 Bax null, were treated with paclitaxel in combination with the ER stress inhibitor, salubrinal, to see if inhibiting ER stress affected the ability of paclitaxel to cause apoptosis. Tunicamycin, an ER stress inducer, acted as a control for the salubrinal. Since tunicamycin was diluted in DMSO, the cells were treated with DMSO as a control. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each drug concentration was averaged to obtain the mean absorbance. The mean absorbance for each drug concentration was then normalized against the mean absorbance of the untreated cells. All tunicamycin treated cells and its combinations were normalized against the DMSO treated cells, while all paclitaxel and salubrinal treated cells and their combinations with each other were normalized against the untreated cells. As observed in Figure 4, both paclitaxel and tunicamycin reduced the number of surviving cells in both cells lines with 23% and 30% cell survival, respectively. Microscopic observation of the cells revealed considerable numbers of dead, floating cells. Co-treatment with salubrinal did not reverse the cell death caused by paclitaxel or tunicamycin, and, in fact, cells treated with salubrinal alone showed a reduction in cell numbers, though not as much as following paclitaxel or tunicamycin treatment.

A **HCT116**



B **Bax null**

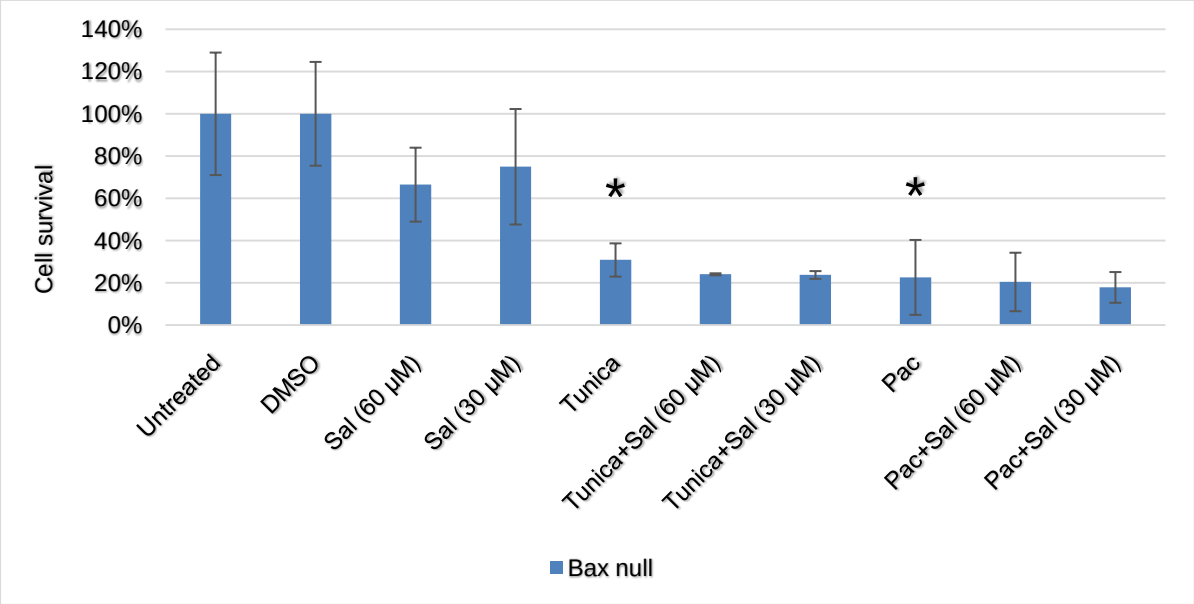


FIGURE 4: Cell survival in colorectal cancer lines after drug treatments.

(A) HCT116 cells were treated with either paclitaxel (32 nM), salubrinal (30 µM, 60 µM), tunicamycin (200 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. (B) HCT116 Bax null cells were treated with either paclitaxel (32 nM), salubrinal (30 µM, 60 µM), tunicamycin (200 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated four times for HCT116 and twice for Bax null, independently of each other. Means ± SEM are shown. T-tests comparing paclitaxel or salubrinal treated cells with untreated cells, or comparing tunicamycin treated cells with DMSO treated cells was performed, and (*) indicates p values ≤ 0.05.

3.4 Establishing conditions for the SKOV3 ovarian cancer cell line

Due to the fact that salubrinal killed the HCT cells, suggesting that they may have inherently activated UPR and therefore did not show induction of the UPR following paclitaxel treatment, it was decided to try using different cell lines. Ovarian cell lines were chosen since previous WWOX studies in our laboratory showing the activation of ER stress by paclitaxel were done using the ovarian PEO1 cell line. Three ovarian cell lines were obtained: A2780, SKOV3 and PEO1. However, problems culturing PEO1 cells limited their use, and so, they were not used in this experiment. To establish appropriate cell numbers for SKOV3 cells, the cell line was plated at various cell densities (in triplicates) and grown for 120 hours. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each cell density was averaged to obtain the mean absorbance. The lowest cell density that reached near full confluence after 120 hours was chosen as the appropriate cell density; this was found to be 6,000 cells (Figure 5). Due to the availability of new spectrophotometric plate readers, SRB staining analysis of cell lines could now be read at the optimum wavelength of 570 nm. To ensure the accuracy of the spectrophotometric analysis, an experiment using SKOV3 cells was set up to optimize the parameters for the technique. For this experiment, SKOV3 cells were plated in various cell densities (in triplicates) and grown for 120 hours. Following SRB staining, spectrophotometric analysis was conducted using both the 492 nm and 570 nm wavelengths to see if there was an actual difference in the accuracy of the readings. As before, the mean background was removed from each absorbance reading and then the triplicates for each cell density was averaged to obtain the mean absorbance. The mean absorbance for each cell density was then normalized against the mean absorbance of the lowest cell density. Figure 5 shows that the overall pattern of the data is the same at 492 and 570, although the fold differences appear slightly less when measured at 570 nm. Subsequent experiments using ovarian cancer cell lines therefore used 570 nm to measure SRB absorbance.

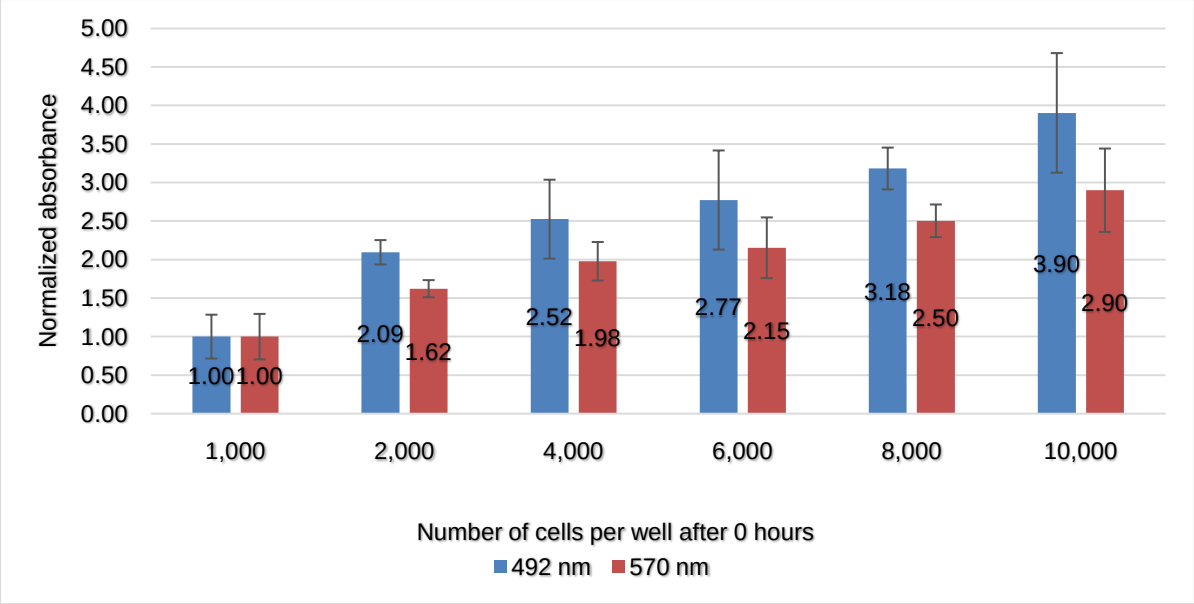


FIGURE 5: Absorbance readings of SKOV3 cells at 492 nm and 570 nm
SKOV3 cells at varying densities were grown for 120 hours and then stained using SRB dye. The absorbance was read at both 492 nm and 570 nm. Bars represent means of triplicate wells ± standard deviation.

For the SRB staining process, the fixed cells (in the wells) are stained with SRB solution and incubated for 30 minutes before being washed and dried. The dried cell bound dye is then re-solubilized in Tris solution before reading the absorbance. So, in addition to the wavelength, we also wanted to see if the fixed cells made a difference in the absorbance reading compared to just reading the re-solubilized dye in a new plate without the cells. To test this, all of the re-solubilized dye was transferred over to a new plate following SRB staining. As before, the mean background was removed from each absorbance reading and then the triplicates for each cell density was averaged to obtain the mean absorbance. The mean absorbance for each cell density was then normalized against the mean absorbance of the lowest cell density. Figure 6 shows that the presence of the fixed cells in the wells made a difference in the readings as the plate with the fixed cells had much higher absorbance readings than the plate with only the Tris re-solubilized dye. Based on the data shown on Figures 5 and 6, all following cell survival experiments were conducted by transferring the Tris re-solubilized dye into a new plate and reading the absorbance at 570 nm.

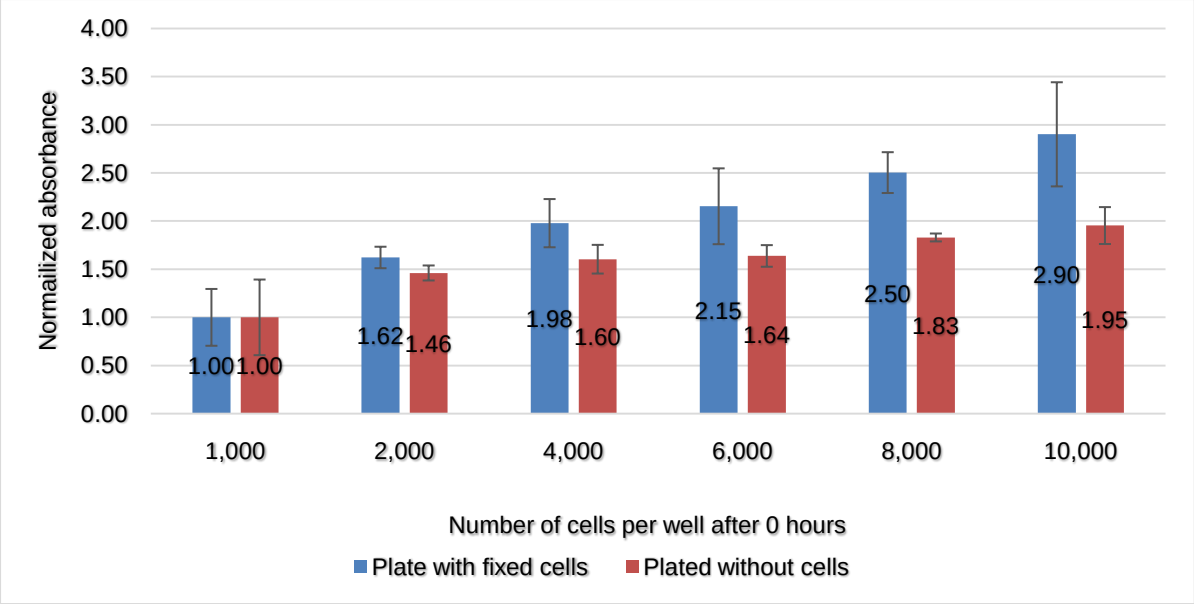


FIGURE 6: Absorbance readings of SKOV3 cells at 570 nm - plate with cells vs. plate with only Tris. SKOV3 cells at varying densities were grown for 120 hours and then stained using SRB dye. 150 µl of the Tris re-solubilized dye was transferred to a new plate and both plates were read at 570 nm. Bars represent means of triplicate wells ± standard deviation.

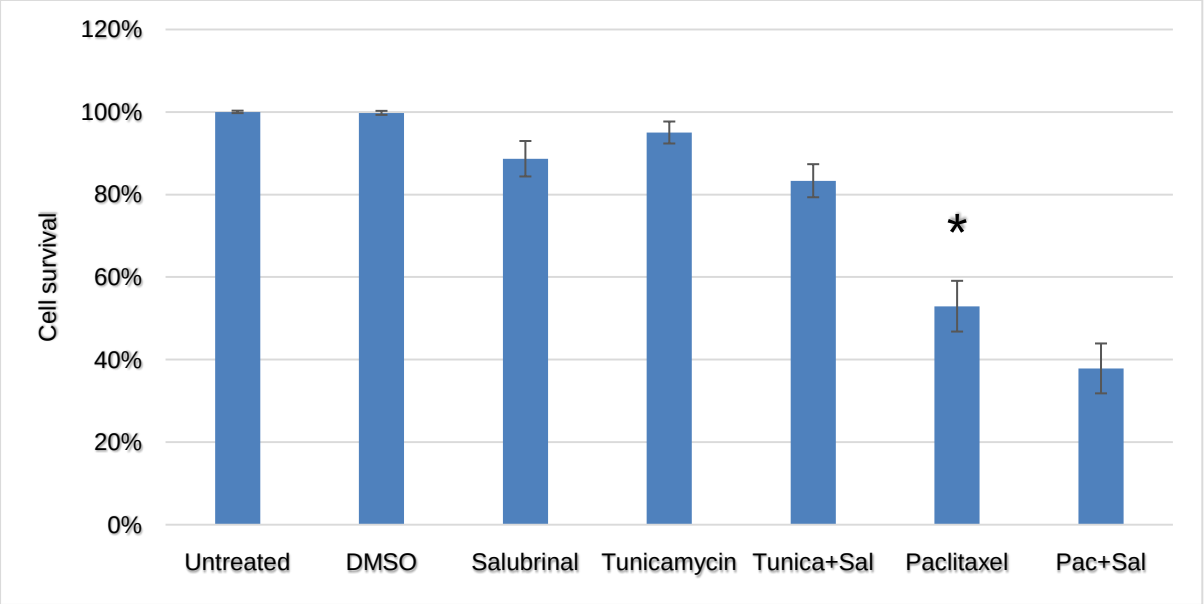
Conditions for the A2780 cell line were previously established by Dr Szymon Janczar, and were found to be 15,000 log-phase cells per well, and 16 nM paclitaxel.

3.5 Paclitaxel treatment and ER stress inhibition in A2780 and SKOV3 cell lines

In this experiment, the ovarian cancer cell lines, A2780 and SKOV3, were treated with paclitaxel in combination with the ER stress inhibitor, salubrinal, to see if inhibiting ER stress affected the ability of paclitaxel to cause apoptosis. Tunicamycin, an ER stress inducer, acted as a control for the salubrinal. Since the tunicamycin was diluted in DMSO, the cells were treated with DMSO as a control. The appropriate cell densities for A2780 (15,000 cells) and SKOV3 (6,000 cells), and the appropriate paclitaxel concentrations to use (16 nM for A2780 and 20 µM for SKOV3) were validated from previous studies as described above. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each drug concentration was averaged to obtain the mean absorbance. The mean absorbance for each drug concentration was then normalized against the mean absorbance of the untreated cells. As shown in Figure 7, paclitaxel reduced the number of surviving cells in both cells lines by 50%. Microscopic observation of the cells revealed considerable numbers of dead, floating cells. Salubrinal alone had only a small effect on cell survival, but it did not reverse the cell death caused by

paclitaxel. Tunicamycin had no effect on cell survival in A2780 and marginal effect in SKOV3; salubrinal did not reverse this effect.

A **A2780**



B **SKOV3**

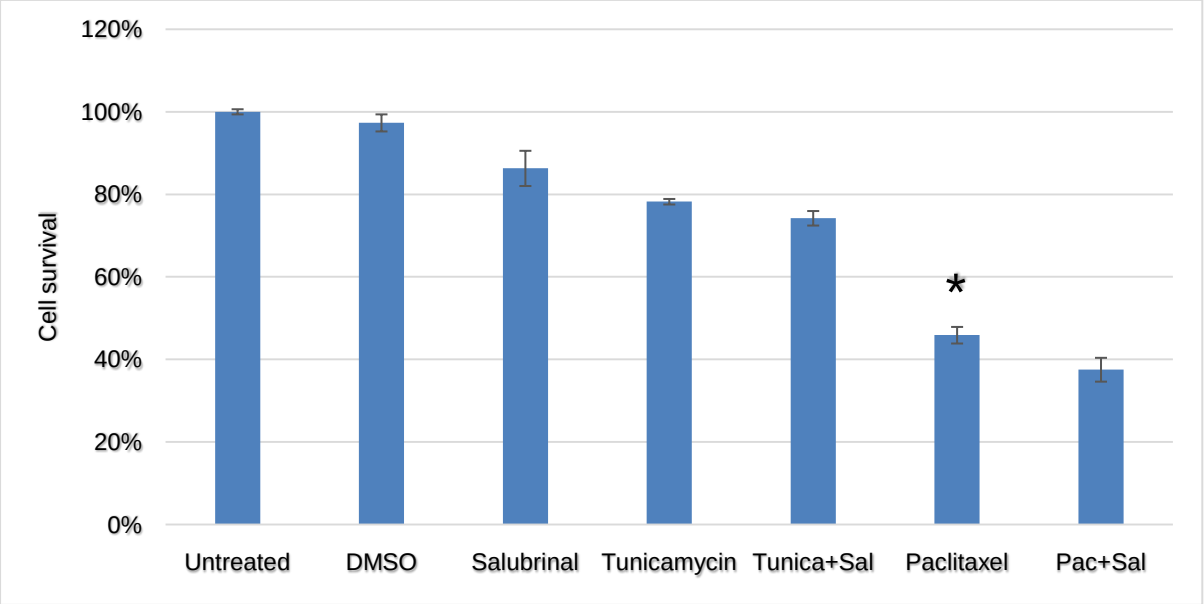
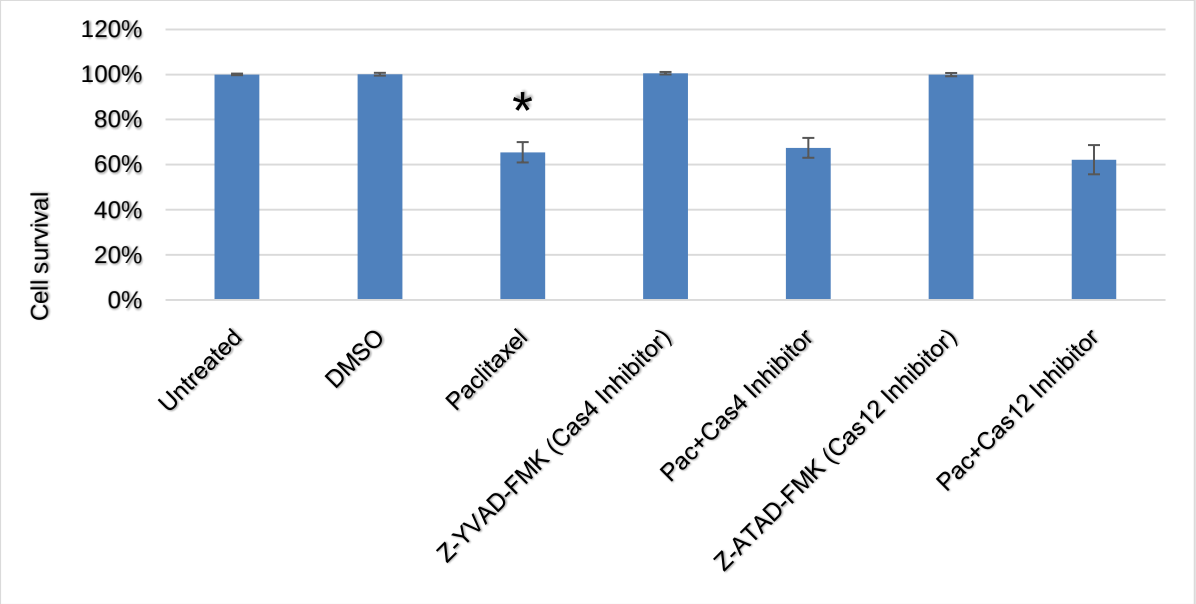


FIGURE 7: Cell survival in ovarian cancer cell lines after ER stress inhibition by salubrinal.
(A) A2780 cells were treated with either paclitaxel (16 nM), salubrinal (30 µM), tunicamycin (200 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. (B) SKOV3 cells were treated with either paclitaxel (20 µM), salubrinal (30 µM), tunicamycin (200 ng/ml), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated five times for A2780 and three times for SKOV3, independently of each other. Means ± SEM are shown. T-tests comparing paclitaxel, tunicamycin or salubrinal treated cells with untreated cells was performed, and (*) indicates p values ≤ 0.05.

Next, the two ovarian cancer cell lines were treated with paclitaxel in combination with caspase-4 or -12 inhibitors, Z-YVAD-FMK and Z-ATAD-FMK, respectively, to see if inhibiting the caspases activated pathways of the UPR which protected the cells against cell death. Since both the caspase inhibitors were diluted in DMSO, the cells were treated with DMSO as a control. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each drug concentration was averaged to obtain the mean absorbance. The mean absorbance for each drug concentration was then normalized against the mean absorbance of the untreated cells. Figure 8 shows that as expected, paclitaxel reduced the number of surviving cells in both cells lines by 50%. Microscopic observation of the cells revealed considerable numbers of dead, floating cells. Inhibiting caspase-4 and -12 did not reverse the cell death caused by paclitaxel, nor did their inhibition in either cell line cause cell death.

A **A2780**



B **SKOV3**

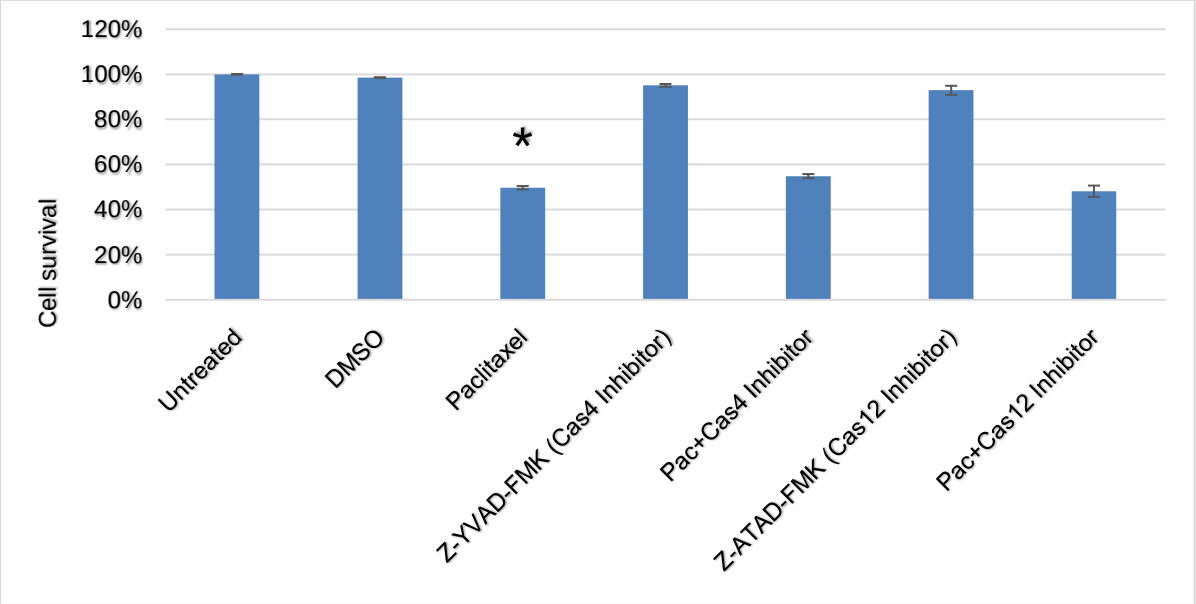
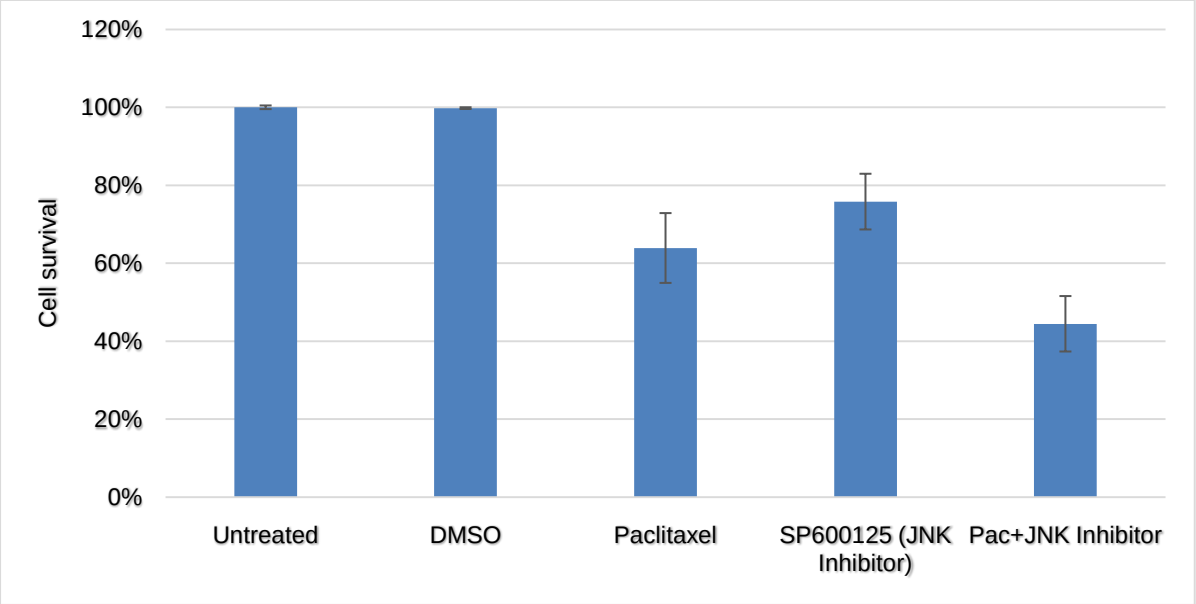


FIGURE 8: Cell survival in ovarian cancer cell lines after caspase-4 and -12 inhibition.
(A) A2780 cells were treated with either paclitaxel (16 nM), Z-YVAD-FMK (10 µM), Z-ATA-FMK (10 µM), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. (B) SKOV3 cells were treated with either paclitaxel (20 µM), Z-YVAD-FMK (10 µM), Z-ATA-FMK (10 µM), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated three times, independently of each other for both cell lines. Bars represent means of triplicate experiments ± SEM. T-tests comparing paclitaxel, caspase-4 or caspase-12 treated cells with untreated cells was performed, and (*) indicates p values ≤ 0.05.

Following the caspase inhibition experiments, the two ovarian cancer cell lines were treated with paclitaxel in combination with the JNK inhibitor, SP600125, to see if paclitaxel used this UPR pathway to induce apoptosis via ER stress. Since the JNK inhibitor was diluted in DMSO, the cells were treated with DMSO as a control. Following SRB staining and spectrophotometric analysis, the mean background was removed from each absorbance reading and then the triplicates for each drug concentration was averaged to obtain the mean absorbance. The mean absorbance for each drug concentration was then normalized against the mean absorbance of the untreated cells. Figure 9 shows that as expected paclitaxel reduced the number of surviving cells in both cells lines. Though by a much greater amount than 50% in the SKOV3 cells. Microscopic observation of the cells revealed considerable numbers of dead, floating cells. Inhibition of JNK did not reverse the cell death caused by paclitaxel, but its inhibition did reduce the number of surviving cells in both cell lines.

A **A2780**



B **SKOV3**

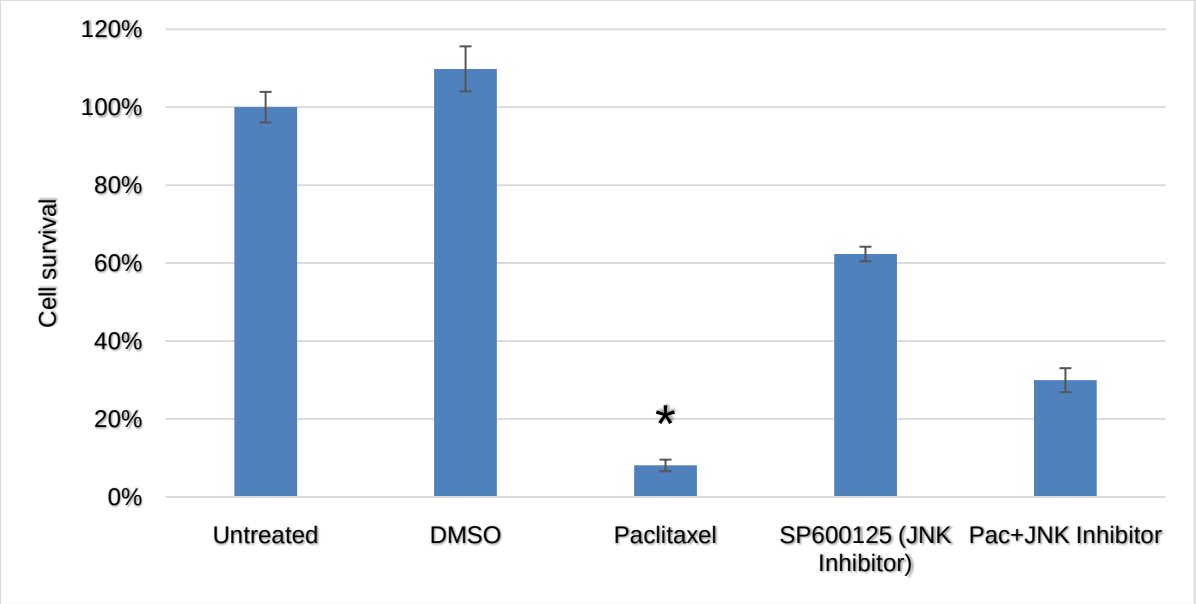


FIGURE 9: Cell survival in ovarian cancer cell lines after JNK inhibition
(A) A2780 cells were treated with either paclitaxel (16 nM), SP600125 (10 µM), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. (B) SKOV3 cells were treated with either paclitaxel (20 µM), SP600125 (10 µM), or in combination with each other. After 72 hours, spectrophotometric analysis was used (post SRB staining) to observe cell survival. Experiments were repeated three times, independently of each other for both cell lines. Bars represent means of triplicate experiments ± SEM. T-tests comparing paclitaxel, or SP600125 treated cells with untreated cells was performed, and (*) indicates p values ≤ 0.05.

3.6 Protein analysis of A2780 cell line via western blot

To test whether paclitaxel was inducing the ER stress pathway in these cell lines and to confirm the inhibitor drugs were in fact working, a time-course experiment using the A2780 cell line was carried out. The cells were treated with paclitaxel, or paclitaxel in combination with either salubrinal, or SP600125 (JNK inhibitor). The treatments were stopped and the cells lysed at specific time intervals ranging from 0-36 hours. Protein analysis of the lysates was conducted via western blot. The membranes produced through the western blots were than probed with antibodies for known protein markers of ER stress. Figure 10 shows the western blots for cells treated with paclitaxel alone with no evidence of ER stress induction following paclitaxel treatment. The housekeeping protein, HSP60 was used to show equal level of protein in the lanes. There seems to be small levels of phospho-eIF2α and phospho-c-Jun at the 4 hour exposure time, however, upon further observation this can be contributed to there being more protein loaded in the lane. No bands were detected for phospho-JNK or cleaved caspase-3. Figure 11 shows the western blots for cells co-treated with paclitaxel and salubrinal with no evidence of change in phospho-eIF2α levels, or other ER stress markers, following salubrinal treatment. Again, the housekeeping protein, HSP60, was used to show equal level of protein in the lanes. There are levels of phospho-c-Jun at the 4 and 8 hour exposure times, and again no bands for phospho-JNK or cleaved caspase-3 were detected. Figure 12 shows the western blots for cells co-treated with paclitaxel and the JNK inhibitor, SP600125, with no evidence of a change in ER stress markers following SP600125 treatment. The housekeeping protein, HSP60, was again used to show equal level of protein in the lanes. There appears to be higher levels of phospho-eIF2α with bands showing at the 2, 4, 8, 24 and 36 hour exposure times. Levels of phospho-JNK can also be observed at the 0 and 8 hour exposure times. Again, bands for phospho-c-Jun and cleaved caspase-3 were not detected. Paclitaxel, salubrinal and SP600125 do not show to alter the expression of the late ER stress marker GRP78.

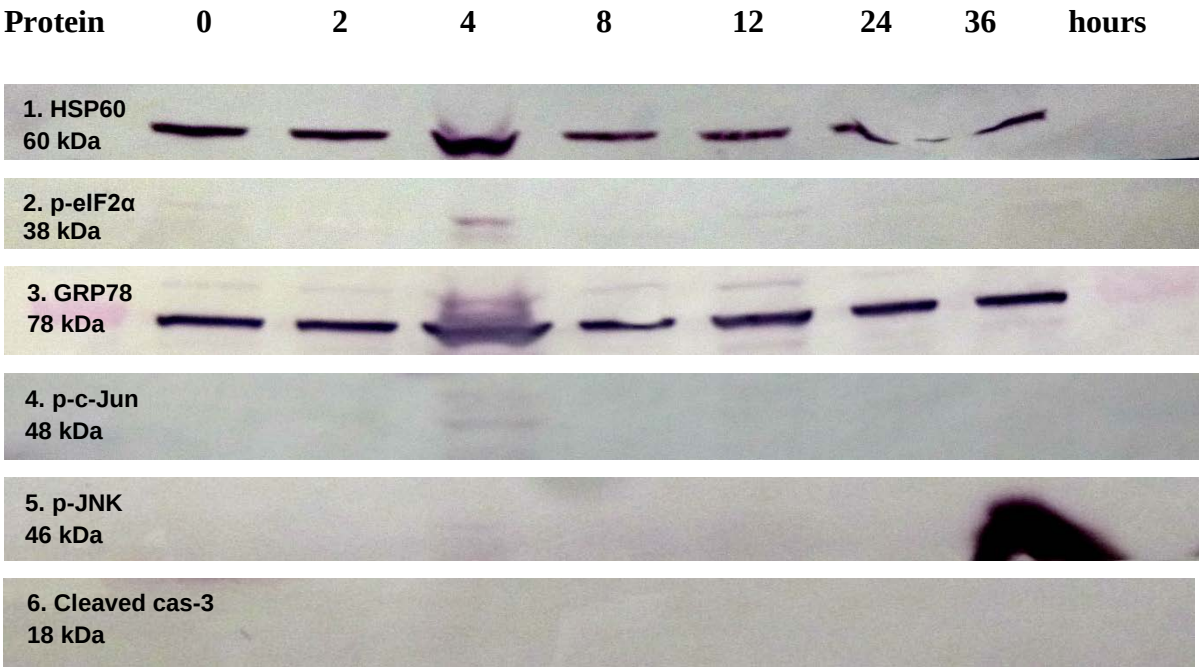


FIGURE 10 Western blots of paclitaxel treated A2780 cell lysates.
1. Confirms that each lane contains equal amounts of protein (equal HSP60 signal). 2. Shows level of phospho-eIF2α. 3. Shows level of GRP78. 4. Shows level of phospho-c-Jun. 5. Shows level of phospho-JNK. 6. Shows level of cleaved caspase-3.

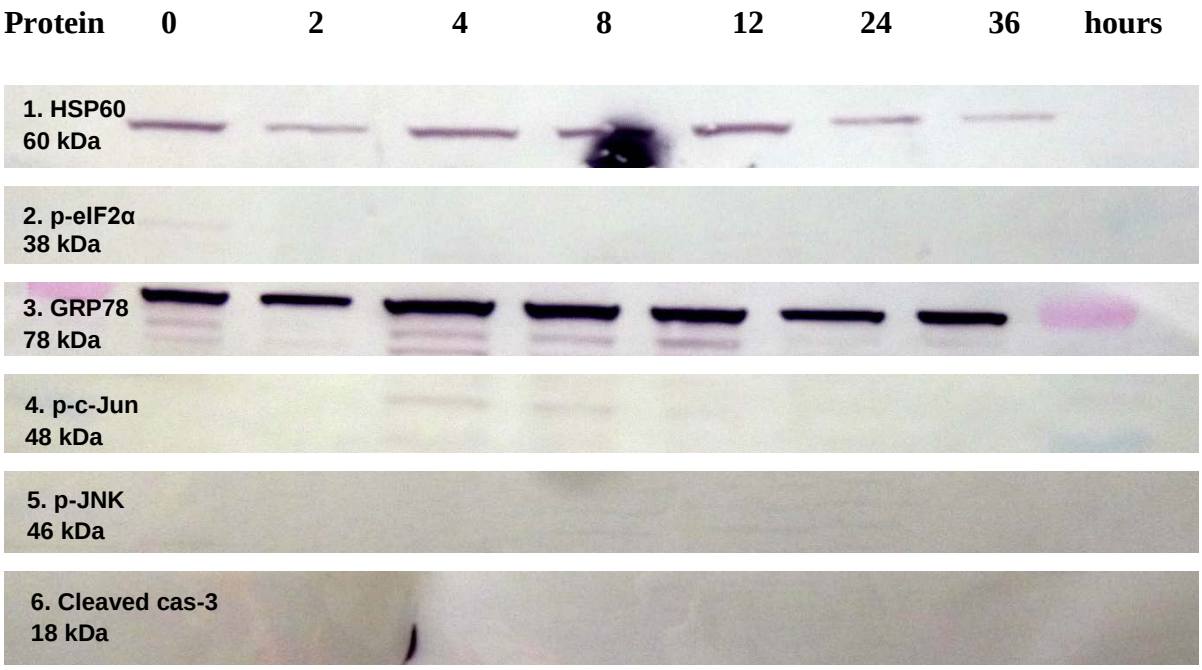


FIGURE 11: Western blots of salubrinal treated A2780 cell lysates.
1. Confirms that each lane contains equal amounts of protein (equal HSP60 signal). 2. Shows level of phospho-eIF2α. 3. Shows level of GRP78. 4. Shows level of phospho-c-Jun. 5. Shows level of phospho-JNK. 6. Shows level of cleaved caspase-3.

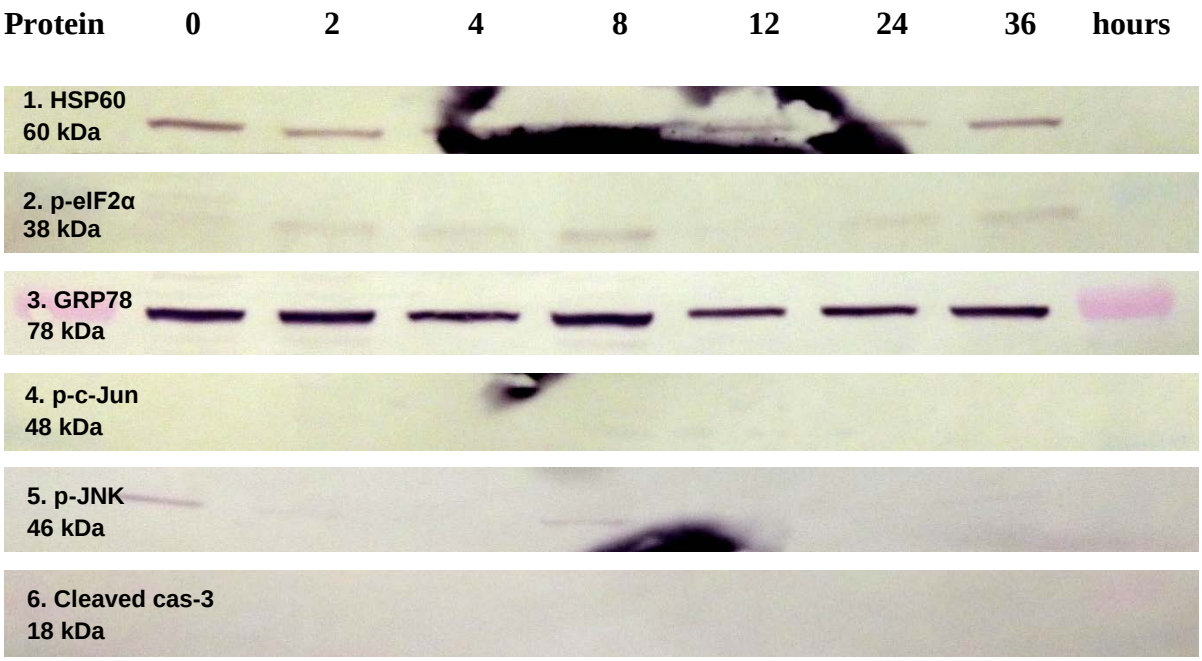


FIGURE 12: Western blots of SP600125 (JNK inhibitor) treated A2780 cell lysates.
1. Confirms that each lane contains equal amounts of protein (equal HSP60 signal). 2. Shows level of phospho-eIF2α. 3. Shows level of GRP78. 4. Shows level of phospho-c-Jun. 5. Shows level of phospho-JNK. 6. Shows level of cleaved caspase-3.

CHAPTER 4: DISCUSSION

4.1 The role of ER stress in paclitaxel response in HCT116 and HCT116 Bax null cell lines

It has been evidenced in previous studies that the chemotherapy drug, paclitaxel, induces cellular apoptosis, in part, via activation of ER stress and the UPR pathway. At the start of this research, this apoptotic mechanism of paclitaxel was tested on two colorectal carcinoma cell lines: HCT116 and HCT116 Bax null. Along with paclitaxel, the cell lines were treated with the ER stress inhibitor, salubrinal, to see whether the inhibition of ER stress affected the apoptotic ability of paclitaxel. Tunicamycin, a known ER stress inducer, was used as a control to test that salubrinal could effectively inhibit ER stress.

From the results (Chapter 3, Section 3.3, Figure 4) it can be seen, that cells treated with paclitaxel showed a lower percentage of cell survival than those cells that were left untreated. In both the HCT116 and HCT116 Bax null cell lines, the amount of surviving cells after paclitaxel treatment was 23%; suggesting that paclitaxel did in fact cause cell death. Similarly, cells treated with tunicamycin showed a much lower percentage of cell survival than those left untreated with approximately a 70% reduction in the number of surviving cells for both cell lines. Salubrinal-only treated cells also showed a reduction in the number of surviving cells with approximately 50% cell survival in the HCT116 cell line and approximately 70% in the HCT116 Bax null cell line. However, statistical analysis showed this to be significant only in the HCT116 cells treated with 60 µM salubrinal. Not surprisingly, salubrinal also did not show to reverse the effects of either paclitaxel or tunicamycin. Both paclitaxel and tunicamycin showed no change in the amount of cell death when in combination with salubrinal. This was validated by t-tests which showed the p-values to be≥ 0.05 when comparing the cell survival of paclitaxel or tunicamycin treated cells to those cells treated with paclitaxel or tunicamycin in combination with salubrinal; proving any change in the amount of cellular death by salubrinal to be insignificant. These findings suggest that perhaps the cells are using ER stress to stay alive and so, inhibiting ER stress kills them.

Perhaps in the HCT116 and Bax null cell lines, inhibition of ER stress causes cell death because ER stress is inherently active and protects the cells against the apoptotic response. As previously mentioned, it has been widely evidenced that the activation of the UPR and its cytoprotective nature play an important role in cancer development by not only helping them

grow, but by also helping them evade the apoptotic arm of the UPR itself (Mann and Hendershot, 2006). If some cancer cells have already evolved and acquired a mutation to have ER stress inherently activated, thus benefiting from use of its anti-apoptotic response to help them proliferate, then the inhibition of ER stress would stop these cytoprotective components of the UPR from allowing further tumorigenicity. So, it may be possible that these HCT colorectal cells have in them inherently activated ER stress, and thus, why inhibiting ER stress with salubrinal caused cellular death. However, since tunicamycin treatment also was shown to kill the cells, presumably via ER stress induction, then this could suggest that the apoptotic response of the UPR can be reactivated with the help of a stimulator.

However, it should also be considered that the results obtained in this experiment might indicate that salubrinal did not cause ER inhibition, but perhaps instead induced ER stress, or possibly even had some other off target effect. This could explain why treating cells with salubrinal caused cellular death, and why the drug was unable to reverse the effects of either paclitaxel or tunicamycin by inhibiting ER stress. Supporting this, in a study conducted by Drexler et al, it was evidenced that salubrinal lacked a cytoprotective effect against ER stress, and instead enhanced a cytotoxic effect. In this study, Bcr-Abl positive and negative leukemic cells were treated with salubrinal to see if it could protect the cells from proteasome inhibitor-mediated ER stress. The data collected from this study showed that salubrinal did not protect the cells by inhibiting ER stress, but instead synergistically enhanced apoptotic cell death by further boosting ER-stress (Drexler, 2009). In addition, the study also looked at if salubrinal could affect the ER stress-mediated cell death caused by the ER stress inducing drug, thapsigargin. This was done to determine whether salubrinal would also prevent classical ER stress induced by thapsigargin since the specific involvement of proteasome inhibitors in ER stress-mediated cell death may be obscured by the influence of these inhibitors on other possible regulatory pathways. The data collected from this revealed that salubrinal also enhanced the toxic effects of thapsigargin, and did not reverse its ER stress induction (Drexler, 2009). Drexler also suggested in his work that the salubrinal-mediated effects were independent from the nature of the ER stressor and was instead due to intrinsic cell type specific differences in the ER stress signalling mechanisms (Drexler, 2009). This could help answer why salubrinal caused cellular death not only in cells treated with only itself, but slightly increased death when in combination with an ER stress inducer like

tunicamycin, and the possible ER stress-mediated pro-apoptotic mechanism used by paclitaxel.

Finally, it should also be pointed out that paclitaxel is known to cause apoptosis via mitotic arrest by binding to tubulin to promote microtubule assembly, and so, it may be that paclitaxel is only killing through mitotic arrest and is not inducing ER stress-related apoptosis in these cells, hence inhibiting the ER stress pathway does not inhibit paclitaxel induced cell death.

4.2 The effect of inhibiting ER stress in response to paclitaxel in A2780 and SKOV3 cell lines

Due to the suggestive evidence from the HCT116 and HCT116 Bax null cell experiments that ER stress may be inherently active in them, or that salubrinal induces ER stress-related apoptosis in these cell lines, this research looked to characterize the activation of ER stress in other cancer cell lines which did not have inherent ER stress. Based on past WWOX and paclitaxel work by Dr Adam Paige and Dr Szymon Janczar using the ovarian cancer cell line PEO1, this research chose to work on ovarian cancer cell lines. The ovarian cell lines: PEO1, A2780 and SKOV3 were chosen, but due to culturing issues with the PEO1 line, it was not used for the purposes of this experiment. As before, the two cell lines, A2780 and SKOV3, were treated with the ER stress inhibitory drug, salubrinal. This helped to identify whether ER stress was inherently activated in the two cell lines. In addition, the chemotherapy drug, paclitaxel, was used to treat the cell lines in order to see if its effect on the cells were altered by inhibiting known components of the UPR. Once again, the ER stress inducing drug, tunicamycin was used as a control to test that salubrinal inhibition was working.

From the results (Chapter 3, Section 3.5, Figure 7) it can be seen, that as expected, cells treated with paclitaxel showed a lower percentage of cell survival than those cells that were left untreated with both the A2780 and SKOV3 showing approximately 50% of cell survival following paclitaxel treatment. However, tunicamycin had very little effect on cell survival in these lines, unlike in the HCT lines, which hopefully means these cells are not dependent on inherent activation of the UPR. Statistically, t-tests validated that there was not a significant change in the percentage of surviving cells between the tunicamycin treated cells and its control, the DMSO treated cells. Salubrinal treated cells once again showed a reduction in the number of surviving cells, however, this was a much smaller effect than seen previously in

the two colorectal cancer cell lines with less than 15% reduction in cell survival for both ovarian cancer cell lines. In fact, both salubrinal and tunicamycin caused nearly the same amount of death in the cells for both cell lines. Again, salubrinal did not show to reverse the effects of either paclitaxel or tunicamycin, and in both cases, the addition of salubrinal to either paclitaxel or tunicamycin showed to cause slightly more cell death.

Interpretation of the data suggests there could be an issue with tunicamycin due to the inefficiency of drug to work as desired, or expected by causing ER stress induced cell death, as was previously observed in the colorectal cell lines. However, this concern was dealt with by repeating the experiments using the two ovarian cancer cell lines with a new batch of tunicamycin, which produced the same results. Alternatively, it can also be argued that the reason there was no significant change in cell survival for tunicamycin treated cells is that even though ER stress may have been induced in the cells by tunicamycin, the activation of the UPR does not lead to apoptosis in these cells, and thus, why inducing ER stress by tunicamycin caused such low cell death since the initial adaptive nature of ER stress and the early cytoprotective arms of the UPR are already helping to keep the cancer cells alive via their anti-apoptotic pathways (Ma and Hendershot, 2004). Whatever the reason for the lack of cell death caused by tunicamycin treatment, it was therefore not possible to use this drug to confirm that salubrinal was effective in inhibiting UPR.

Though it cannot be said with certainty whether paclitaxel uses UPR activation to induce apoptosis, or whether this is being inhibited by salubrinal, since tunicamycin-related ER stress could not be used as a control for salubrinal-related ER inhibition, what can ultimately be taken from the salubrinal experiments is that paclitaxel does show to clearly induce cell death.

Next, this research looked at inhibition of other known components of the UPR in order to see how this affected paclitaxel response in cells. The two ovarian cell lines were treated with inhibitors for caspase-4, caspase-12 and JNK in addition to paclitaxel. These three enzymes have all been suggested to play roles in ER stress-induced apoptosis (Mhaidat et al., 2011). Caspase-12 is thought to be a key mediator as caspase-12 involvement has been observed in both the mitochondria-dependent and independent pathway of ER stress-induced apoptosis in mice (Lit et al., 2011; Ozcan and Tabas, 2012). Caspase-4 and JNK have been shown to be involved in the IRE1 pathway of the UPR, with TRAF2 promoting the activation of JNK (Ozcan and Tabas, 2012).

From the results (Chapter 3, Section 3.5, Figures 8 & 9) it can be observed that once again cells treated with paclitaxel showed a lower percentage of cell survival than those cells that were left untreated with approximately 50% of cell survival for both cell lines. However, this is not seen in the JNK inhibitor experiment (Figure 9A) where SKOV3 cells treated with paclitaxel showed a far greater amount of cell death, with more than a 90% reduction in cell survival. This was unexpected, as the same dose of paclitaxel (20 µM) consistently caused approximately 50% cell survival in multiple previous experiments with this cell line. This may indicate that an error was made in the drug preparation and a higher than intended dose was used. However, the experiment was repeated three times and consistently showed the same result, and this possibility was further investigated through the western blot experiments discussed below. Cells treated with only Z-YVAD-FMK or only Z-ATAD-FMK (caspase-4 and -12 inhibitors, respectively) showed minor to no change in cell survival in comparison to the untreated cells. In contrast, A2780 cells treated with SP600125 (JNK inhibitor) showed nearly a 25% reduction in cell survival when compared to untreated cells, while SKOV3 cells had nearly a 40% reduction. Following t-tests showed this reduction in A2780 cells to not be statistically significant however, with a p-value of 0.07. Whilst the reduction in cell survival following JNK inhibition in SKOV3 cells was statistically significant with a p-value of 0.003, suggesting that JNK inhibition did cause cell death. Caspase-4 and -12 inhibition did not show to have any effect on paclitaxel response in either cell line; giving almost the same amount of cell death as paclitaxel-only treated cells. JNK inhibition did show to have an effect on paclitaxel response in SKOV3 cells, causing approximately 20% more cells to survive than when treated with paclitaxel alone. However, given the unusually low cell survival caused by paclitaxel in this experiment, these results should be taken with caution.

As mentioned, interpretation of the data does suggest a problem with the concentration of paclitaxel used on the SKOV3 cell line during the JNK inhibition experiments which gave a staggering amount of cell death. The inability of caspase-4 and -12 inhibitors to have an effect on paclitaxel response in either cell lines however could possibly be due to the inefficiency of the inhibitor drugs to inhibit their target proteins. It has been shown that caspase-12 is expressed only in rodents, with its human homologue being silenced by several mutations during evolution. Caspase-4 has been shown to have taken over the function of caspase-12 in humans (Mhaidat et al., 2011). This could then give reason to why caspase-12 showed no effect on paclitaxel since it would not have been in active form in these human ovarian cell lines; though this still does not account for caspase-4 to not have an effect.

Similarly, as stated earlier, JNK inhibition also did not show any effect on paclitaxel. Instead, any cell death observed maybe due to some non-ER stress related mechanism employed by paclitaxel. Since the data from these experiments did not show any significant reversal of paclitaxel effect by any of the inhibitors of the UPR components, it was important to confirm whether the UPR is active in these cell lines (either inherently or induced by paclitaxel), and whether these various inhibitors are effectively inhibiting their targets, and so, western analysis of these proteins became the next logical step in this research.

4.3 Expression levels of ER stress markers following ER stress inhibition and paclitaxel treatment

Since the inhibitory experiments did not reverse the effect of paclitaxel on the ovarian cell lines, this research carried out tests to see whether the drugs were functioning by seeing the expression levels of known ER stress markers: phospho-eIF2α, phospho-c-Jun, GRP78, phospho-JNK, and cleaved caspase-3. A time-course experiment using the A2780 cell line was carried out. The cells were treated with either paclitaxel, or paclitaxel in co-treatment with salubrinal, SP600125 (JNK inhibitor), Z-YVAD-FMK (caspase-4 inhibitor) or Z-ATAD-FMK (caspase-12 inhibitor). Following protein extraction from the drug treated cells over a course of 36 hours, the lysates were analysed via western blot and probed with antibodies for one of the ER stress proteins listed above. Though there were blots produced from cells treated with caspase-4 and -12 inhibitors, we were ultimately unable to use them due to the primary antibodies for caspase-4 and -12 not working. Cleaved caspase-3 was chosen as it is the terminating caspase involved in the apoptotic pathway and therefore its expression would than indicate the induction of the UPR.

From the results it can be observed that in the western blots for the paclitaxel treated cells (Figure 10) there were no signs of ER stress induction since there appears to be no change in the level of expression for the ER stress markers. Though there are faint bands at the 4 hour exposure time for both phospho-eIF2α and phospho-c-Jun, closer inspection of the 4 hour wells for the HSP60 and GRP78 stained blots show there to be more protein loaded on those lanes, and that the detection of the bands for phospho-eIF2α and phospho-c-Jun may be just due to this. In the western blots for the cells co-treated with paclitaxel and salubrinal (Figure 11) there is, again, no evidence of ER stress induction since there appears to be no change in the expression levels for any of the ER stress markers. Faint bands for phospho-c-Jun can be seen at the 4 and 8 hour exposure times, however, this can once again be contributed to the

overloading of protein in the lanes. In the western blots for the cells co-treated with paclitaxel and the JNK inhibitor, SP600125, there is once again no evidence of ER stress induction since there is no change in ER stress marker levels. Even though there seems to be appearances of some faint bands for phospho-eIF2α and phospho-JNK, their expression levels to not steadily increase over time as would be expected in case of ER induction. In addition to not showing any evidence of ER stress induction, all the blots also seem to show no change in the expression level of the ER-stress mediated chaperone, GRP78, or any expression of cleaved caspase-3.

In light of the evidence gathered, the following aspects can be addressed. First, the UPR is not inherently activated in this cell line since there was no evidence to suggest that there was ER stress induction. This then also suggests that paclitaxel did not activate the UPR via ER stress induction since there appears to be no change in the expression levels for any ER stress markers. If the UPR was inherently induced in this cell line, or if paclitaxel caused UPR activation via ER stress induction then we would expect to see changes in the expression levels for the ER stress markers when a specific ER-related protein or pathway was inhibited by one of the inhibitory drugs. For example, if salubrinal was used to inhibit the ER stress induced in the cells (whether inherently activated, or an effect of paclitaxel treatment), then we would expect to see increased levels of phospho-eIF2α over time since salubrinal would stop the PP1a/GADD34 complex from dephosphorylating eIF2α and therefore prolong the reduction of protein synthesis (Drexler, 2009). If SP600125 was used to inhibit JNK during ER stress induction then we would expect to see decreased levels of phospho-c-Jun since JNK would be unable to phosphorylate c-Jun due to its inhibition. In addition, JNK inhibition would show decreased levels of phospho-JNK over time as phosphatases would keep JNK inhibited through dephosphorylation. In case of UPR activation, we would expect to see increased expression for the ER stress chaperone, GRP78, and decreased expression in case of inhibition. A study conducted by Mhaidat et al. has shown that the inhibition of GRP78 before paclitaxel treatment induces the activation of a caspase cascade including caspase-3 (Mhaidat et al., 2011). This suggests that inhibiting GRP78 in these cells could possibly show a higher expression level for cleaved caspase-3. We are not seeing UPR activation in this cell line because ER stress is not inherently activated in them, and instead of inducing ER stress, paclitaxel may be using its mitotic arrest mechanism to induce cell death.

4.4 Conclusion

This research set out to investigate whether paclitaxel treatment induced an ER stress-mediated response by aiming to accomplish two tasks: target ER stress in order to inhibit paclitaxel chemotherapy response, and characterize the activation of ER stress by paclitaxel in different cancer cell lines. In carrying out these aims and collecting the results obtained from the experiments, the following conclusions were drawn: first, paclitaxel caused cell death in both the ovarian and colorectal cell lines; second, salubrinal did not reverse the effect of paclitaxel on the cell lines; third, ER stress maybe inherently activated in the HCT lines; and finally, western blots of A2780 showed no evidence of UPR induction.

It is clear from the results that further investigation will be needed to understand and identify the components of the UPR that are important for paclitaxel response. One way of doing this is by first identifying which cell lines have inherent activation of ER stress and which do not. One possible way of doing this is by designing an experiment using RT PCR (reverse transcriptase polymerase chain reaction) to detect expression of genes known to be involved in ER stress and UPR activation. The method would involve growing cell lines and treating it with and without paclitaxel, and then extracting RNA from the cells. The cDNA produced from the RNA can then be used in RT PCR to amplify the expression of a gene of interest, in this case a gene involved in ER stress. Two possible approaches are to examine by RT PCR the induction of GRP78 and the splicing of XBP1 following paclitaxel treatment, which are both unique to the UPR mechanism. Treating the cell line with paclitaxel would allow us to see which cell lines had an inherent activation of ER stress, and which showed induction of ER stress following paclitaxel treatment.

Cell lines showing UPR activation after paclitaxel treatment could be used to investigate the components of the UPR that are important for how paclitaxel kills cells, by using ER stress inhibiting/inducing drugs, or siRNA targeting key components of UPR to modulate paclitaxel response. The data could then potentially be used to apply similar methods to test the effects of ER stress in other chemotherapy drugs used to treat various other cancer types. This information would greatly benefit cancer patients, as it could enable personalised chemotherapeutic treatments for individual patients suffering from the same or different cancer type by first screening patients to identify those patients likely to respond to and those likely to be resistant to paclitaxel, or other chemotherapy drugs. In addition it could identify possible targets for future drugs which could be combined with paclitaxel to increase its

efficacy. This personalised care could then abolish the need to use the current standard set of chemotherapy drugs available, and reduce the often unnecessary and complicating side-effects involved with such treatments; helping to improve patient survival and minimise the time wasted delivering ineffective treatments.

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APPENDIX 1:

Antibodies used for Western Blotting

Antigen	Species	Supplier	Catalogue No.	Western Blot Dilution	Secondary	Secondary Dilution
cleaved caspase-3	rabbit	R&D	MAB835	1:1000	Anti-rabbit	1:1000
GRP78	mouse	R&D	MAB4846	1:20000	Anti-mouse IGG	1:1000
HSP60	mouse	Abcam	ab46798	1:20000	Anti-rabbit	1:1000
phospho(Ser51)-eIF2α	rabbit	Cell Signalling	97215	1:1000	Anti-rabbit	1:1000
phospho-c-Jun	rabbit	Cell Signalling	32705	1:1000	Anti-rabbit	1:1000
phospho-JNK	rabbit	R&D	AF1205	1:1000	Anti-rabbit	1:1000