Activating Transcription Factor 3 as a regulator and predictor of cisplatin response in human cancers

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Abstract

Platinum-based chemotherapies are effective agents in the treatment of a wide variety of human cancers. However, patients with recurrent disease can become resistant to platinum-based chemotherapy, leading to low overall survival rates. Activating transcription factor 3 (*ATF3*) is a stress-inducible gene that is a regulator of cisplatin-induced cytotoxicity. ATF3 protein expression was upregulated after cytotoxic doses of cisplatin treatment in a panel of cell lines. A chromatin immunoprecipitation assay showed that upon treatment with cisplatin, ATF3 directly bound to the *CHOP* gene promoter and this correlated with an increase in CHOP protein expression. In a 1200 compound library screen performed on cancer cell lines, disulfiram, a dithiocarbamate drug, was identified as an enhancer of the cytotoxic effects of cisplatin. This increased cytotoxic action was likely due to disulfiram and cisplatin's ability to induce ATF3 independently through two separate mechanisms, namely the MAPK and integrated stress pathways. Furthermore, ATF3 protein and mRNA levels were variable amongst human ovarian and lung cancer tissues, suggesting the potential for basal expression of ATF3 to be predictive of cisplatin treatment response. Thus, understanding ATF3's role in cisplatin-induced cytotoxicity will lead to novel therapeutic approaches that could improve this drug's efficacy.

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List of Abbreviations

ALDH	Aldehyde dehydrogenase
AP-1	Activating protein -1
APAF-1	Apoptotic protease activating factor-1
ATF	Activating transcription Factor
ATM	Ataxia telangiectasia mutated
ATP7B	Copper transporting P-type adenosine triphosphate
ATR	ATM and Rad3 related
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology3
BiP	Immunoglobin heavy-chain-binding protein
bZip	Basic region leucine zipper
cAMP	Cyclic adenosine monophosphate
c-Abl	v-abl Abelson murine leukaemia viral oncogene homologue
CCL4	Chemokine C-C motif ligand 4
CDDP	Cisplatin
CDK	Cyclin dependent kinase
ChIP	Chromatin Immunoprecipitation
CHOP	C/EBP homologous protein
CI	Combination index
Cisplatin	Diamminedichloroplatinum (II)
CRE	cAMP responsive element
CREB	CRE Binding
CTR1	Copper transporter -1
eIF2α	eukaryotic initiation factor 2 α
DISC	Death-inducing signalling complex
DSB	Double strand breaks
DTT	1,4-dithio-DL-threitol
E2F1	E2F transcription factor 1
eIF2	Eukaryotic initiation factor 2
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERCC1	Excision repair cross-complementing-1
ERK	Extracellular signal-regulated kinase
Erg-1	Early growth response
F2RL2	Coagulation factor II receptor-like 2
Fa-CI	Fraction affected-combination index
FADD	Fas-associated death domain
FasL	Fas-ligand
FDA	Food and drug administration
GADD153	Growth arrest DNA damage inducible gene 153
GAPDH	Glyceraldehyde 3-Phosphate dehydrogenase
GCN2	General control non-derepressible-2
GRP78	78-kDa glucose-regulated protein
HDAC	Histone deacetylase
HNSCC	Head and neck squamous cell carcinoma
hGal9	Galectin-9
HIF	Hypoxia-inducible factor
HMGB	High-mobility group box
HRI	Heme-regulated inhibitor
HRP	Horseradish peroxidase
Id-1	Inhibitor of different
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IR	Ionizing radiation
ISR	Integrated stress response
JNK	c-Jun N-terminal protein kinase
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute
MMR	Mismatch repair
MMS	Methyl methanesulphonate
mRNA	messenger RNA

MRP2	Multidrug resistance protein 2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
NCI	National Cancer Institute
NER	Nucleotide excision repair
NSCLC	Non-small cell lung cancer
OHRI	Ottawa Hospital Research Institute
P21	$P21^{WAF\_1/Cip1}$
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate buffered saline
PERK	PKR-like ER kinase
PMSF	Phenylmethylsulfonyl fluoride
Pol-β	DNA polymerase-β
PTEN	Phosphatase and tensin homolog
PUMA	P53-upregulated modulator of apoptosis
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SAPK	Stress activated protein kinase
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interference RNA
TBS	Tris buffered saline
TBS-T	TBS with 0.1% Tween-20
TGF-β	Transforming growth factor-β
TLR-4	Toll-like receptor-4
TMA	Tissue microarray
TNF-α	Tumour necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
UTR	Untranslated region
UV	ultraviolet light
XIAP	X-linked inhibitor of apoptosis protein
XP	Xerodermal pigmentosum
XPA	XP complementation group A
XPF	XP complementation group F

Chapter 1: Introduction

1.1. Cisplatin as an anti-cancer agent

Cis-Diamminedichloroplatinum (II) (cisplatin) is currently one of the most widely used anti-cancer drugs. It is part of the treatment modality for a wide range of solid tumours including ovarian and non-small cell lung cancer (NSCLC) and it is undergoing clinical trials to assess activity in prostate and breast cancer (1-5). Ovarian cancer has the highest death rate of all gynaecologic cancers mainly due to diagnosis in late stage disease (6). After debulking surgery, first-line therapy for advanced ovarian cancer consists of platinum combination chemotherapy. However, only 25-30% of patients have a complete response to this therapy and second-line treatment options are rarely curative (6). NSCLC accounts for 85% of all lung cancers, the leading cause of cancer-related death in the United States (7). Similar to ovarian cancer, NSCLC is often detected at advanced stage disease due to a lack of effective screening methods (7, 8). Platinum combination chemotherapy is the standard of care for patients with advanced NSCLC although response rates are low (17-32%) and there is only a small prolongation of survival (7). Clearly, enhancing the efficacy of cisplatin therapy is critical for improving the survival of these patient populations.

The use of platinum therapy is showing promise in two other cancer types. Prostate cancer, the most common cancer among men, is currently treated by first-line androgen-deprivation therapy although patients who become resistant to this treatment have few second-line chemotherapy options (5, 9). Satraplatin, a platinum-based agent that is structurally similar to cisplatin and acts by the same mechanism of cytotoxicity, is currently in clinical trials for use as second-line chemotherapy in metastatic castrate-resistant prostate cancer (10). Breast cancer, the most common malignancy among women, is a heterogeneous disease comprised of different sub-

types, all with different treatment options (4). DNA-damaging agents, such as cisplatin, are new treatment options for breast tumours with DNA repair dysfunction due to BRCA1 mutation or downregulation (3). Neoadjuvant, single-agent cisplatin showed high rates of pathological complete response in breast cancer patients with BRCA1-defective tumours (3, 4). Therefore, platinum-based drugs are an important class of anti-cancer agents that merit further research in order to improve their efficacy in a number of different cancers.

Cisplatin was serendipitously discovered in 1965 by the biophysicist Barnett Rosenberg from Michigan State University (11). With the intent of studying cell division, he applied platinum electrodes to bacteria and observed that a by-product, the cisplatin molecule, changed their conformation and inhibited their growth. In 1968, cisplatin was shown to be effective against a murine tumour model (12), which led to the first patient treatment in 1971 and Food and Drug Administration (FDA) approval for testicular and bladder cancer in 1978 (13). In the 1980s, there was a great impetus for the discovery of molecules with safer toxicity profiles. Carboplatin, a derivative of cisplatin with a more stable leaving group, was approved in 1989 for the treatment of ovarian cancer (13). Carboplatin's rate of DNA binding is 10-fold slower than cisplatin and even though 20 - 40 times higher concentrations must be administered, the risk of nephrotoxicity and neurotoxicity is greatly reduced (13). Since then, little progress has been made in developing new platinum drugs with enhanced efficacy. This is mostly due to a lack of understanding of the mechanisms involved in platinum-induced cytotoxicity. Advances in the field of platinum research will allow the development of targeted combination therapies with the goal of avoiding toxic side effects and overcoming tumour resistance.

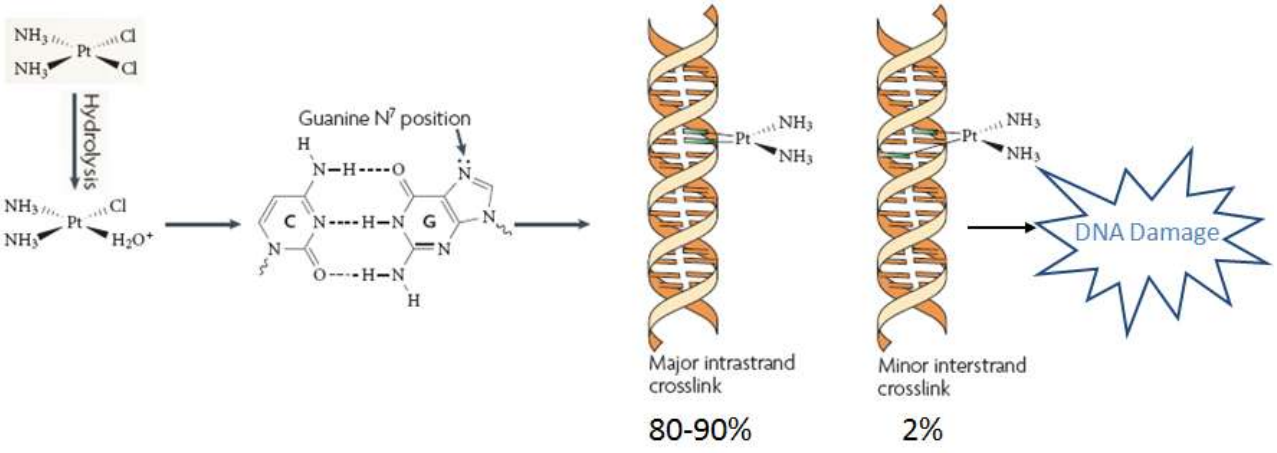
The most widely studied mechanism of cisplatin cytotoxicity is its ability to damage DNA. In the blood stream, cisplatin is a neutral molecule that can freely diffuse through the plasma

membrane (14). Once inside the cell, the cytoplasmic chloride concentration is less than 100mM and cisplatin hydrolysis occurs spontaneously (14, 15). Cisplatin's chloride ions are replaced with water molecules forming a positively charged electrophile that can interact with negatively charged DNA, RNA and proteins (1). Approximately 5 - 10% of intracellular cisplatin molecules irreversibly bind DNA at position N7 of guanine and adenine bases (1, 16, 17). The most common adducts are 1,2-intrastrand crosslinks, which are formed primarily between guanosine residues and lead to bending and partial unwinding of the double helix (Figure 1.1). Damage recognition proteins bind the cisplatin-DNA adduct to either repair it by the nucleotide excision repair (NER) pathway or shield and stabilize the lesion, which results in DNA damage (17). DNA adduct formation is thought to be responsible for inducing apoptosis in cancer cells because transplatin, the trans analogue of cisplatin, cannot form 1,2-intrastrand crosslinks and is biologically inactive (14). As well, NSCLC patients who respond better to cisplatin treatment were found to have a higher proportion of 1,2-intrastrand crosslinks than non-responders (18). However, the molecular pathway linking DNA damage to apoptosis is not completely understood and research is ongoing (13, 19).

1.2. Mechanisms of DNA damage-induced apoptosis

Cisplatin covalently binds DNA to form bulky adducts that block replication and transcription, which leads to G2 phase cell cycle arrest (13). However, DNA platination alone does not correlate with cytotoxicity (17). It is the recognition of DNA damage by a host of proteins which activate pro-survival and pro-apoptotic signals that determine the cell's fate (20).

Figure 1.1. Mechanism of cisplatin-DNA adduct formation. The cisplatin molecule undergoes hydrolysis upon entering the cell whereby a chloride ion is replaced by a water molecule. The newly formed electrophile interacts primarily with guanine residues at position N7 to form intrastrand (80-90% of all crosslinks) and interstrand (2% of all crosslinks) crosslinks. Adapted and modified from Kelland, 2007 (13).


Hydrolysis
Guanine N7 position
Major intrastrand crosslink
80-90%
Minor interstrand crosslink
2%
DNA Damage

1.2.1. P53-dependent mechanisms

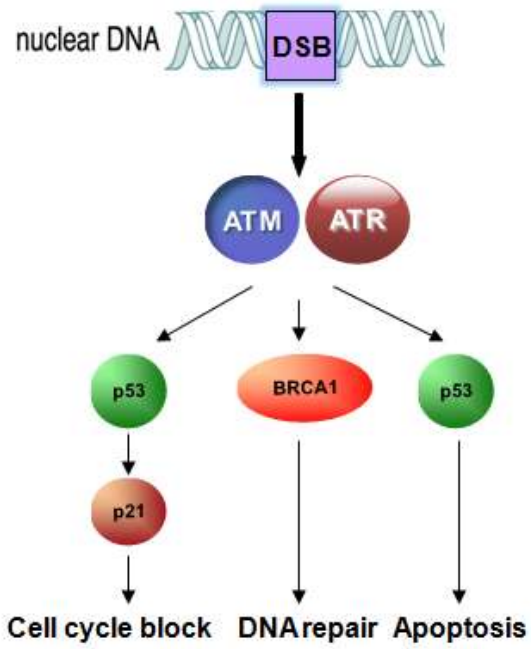
High-mobility group box (HMGB) proteins specifically recognize and bind sites of cisplatin-DNA adducts to shield them from DNA repair mechanisms (17). Studies have shown that high HMGB protein levels correlate with reduced nucleotide excision repair and increased cisplatin sensitivity (17). If cisplatin adducts are not repaired prior to DNA replication, the replication fork will stall at the lesion and the unreplicated single stranded DNA will break to cause DNA double strand breaks (DSB) (21). DSB caused by cisplatin are recognized by the DNA damage checkpoint kinase proteins ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR), which phosphorylate downstream substrates involved in cell cycle control, DNA repair and apoptosis (Figure 1.2A) (22). A low-level activation of p53 is sufficient to induce cyclin-dependent kinase inhibitor $p21^{WAF\_1/Cip1}$ (p21), which disrupts the cell cycle by triggering G1→S checkpoint arrest (17). If the damage is irreparable, sustained protein levels of p53 activate apoptosis by repressing the transcription of anti-apoptotic B-cell lymphoma (Bcl)-2 proteins and by signalling the pro-apoptotic Bcl-2-associated X (BAX) protein to translocate from the cytosol to the mitochondria (17, 20).

1.2.2. Stress-mediated pathway

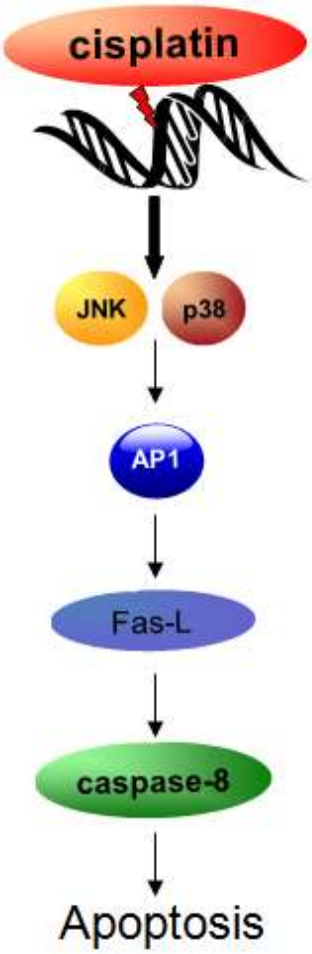
The mitogen-activated protein kinase (MAPK) cascade is an extracellular stress response pathway that includes three kinase members: extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK) also called stress activated protein kinase (SAPK), and p38; all of which are activated by cisplatin (20). This pathway is important for the cytotoxic action of cisplatin since inhibiting any member can attenuate cisplatin-induced apoptosis (23, 24). Ovarian cancer cells display JNK and p38 kinase activation immediately after cisplatin treatment

Figure 1.2. Mechanisms of DNA damage-induced apoptosis. **A**, Double strand breaks (DSB) activate the ATM and ATR proteins, which phosphorylate downstream targets involved in cell cycle control, DNA repair, and apoptosis. **B**, Cisplatin-DNA adducts trigger the long-term activation of JNK and p38 pathways, which mediate the activation of AP-1. AP-1 transcribes Fas-L, which leads to death receptor activation and accumulation of activated caspase 8. Caspase 8 signals apoptosis initiation. Adapted and modified from Roos 2006 (22).

A


nuclear DNA
DSB
ATM
ATR
p53
BRCA1
p53
p21
Cell cycle block
DNA repair
Apoptosis

B


cisplatin
JNK
p38
AP1
Fas-L
caspase-8
Apoptosis

and their expression lasts for several days (25). Once activated, JNK and p38 phosphorylate activating protein -1 (AP-1), which is a dimer of c-Fos and c-Jun (Figure 1.2B) (22). Roos *et al.* postulate that activated AP-1 is protecting the cell by stimulating DNA repair gene expression after initial cisplatin exposure (22). Only when proper DNA repair fails and there is sustained activation of the MAPK pathway, does apoptosis initiate (25). This occurs through the transcriptional activation of Fas-ligand (FasL) after two to four days of cisplatin exposure (22).

1.2.3. Apoptosis independent of nuclear DNA damage

Recently, cisplatin was shown to induce apoptosis independently of nuclear DNA damage through the activation of the endoplasmic reticulum (ER) stress pathway (19). In this study, cisplatin-treated enucleated cells activated an ER-specific caspase that participates in ER stress-induced apoptosis. The caspase was not specifically identified since a mouse antibody with specificity to both human caspase 12 and human caspase 4 was used. Another study found that cisplatin formed 300 - 500 more platinum adducts with mitochondrial DNA than with nuclear DNA (26). Cells with ethidium bromide-depleted mitochondrial DNA were 4 - 5 times more resistant than the parental controls and isolated mitochondria induced immediate apoptosis through cytochrome c release (26).

The balance of pro- and anti-apoptotic protein expression in response to cisplatin treatment will determine whether a cell undergoes DNA repair or succumbs to apoptosis. Cisplatin has pleiotropic effects on the cell and investigating cellular pathways of cisplatin cytotoxicity may lead to the development of novel targeted therapies.

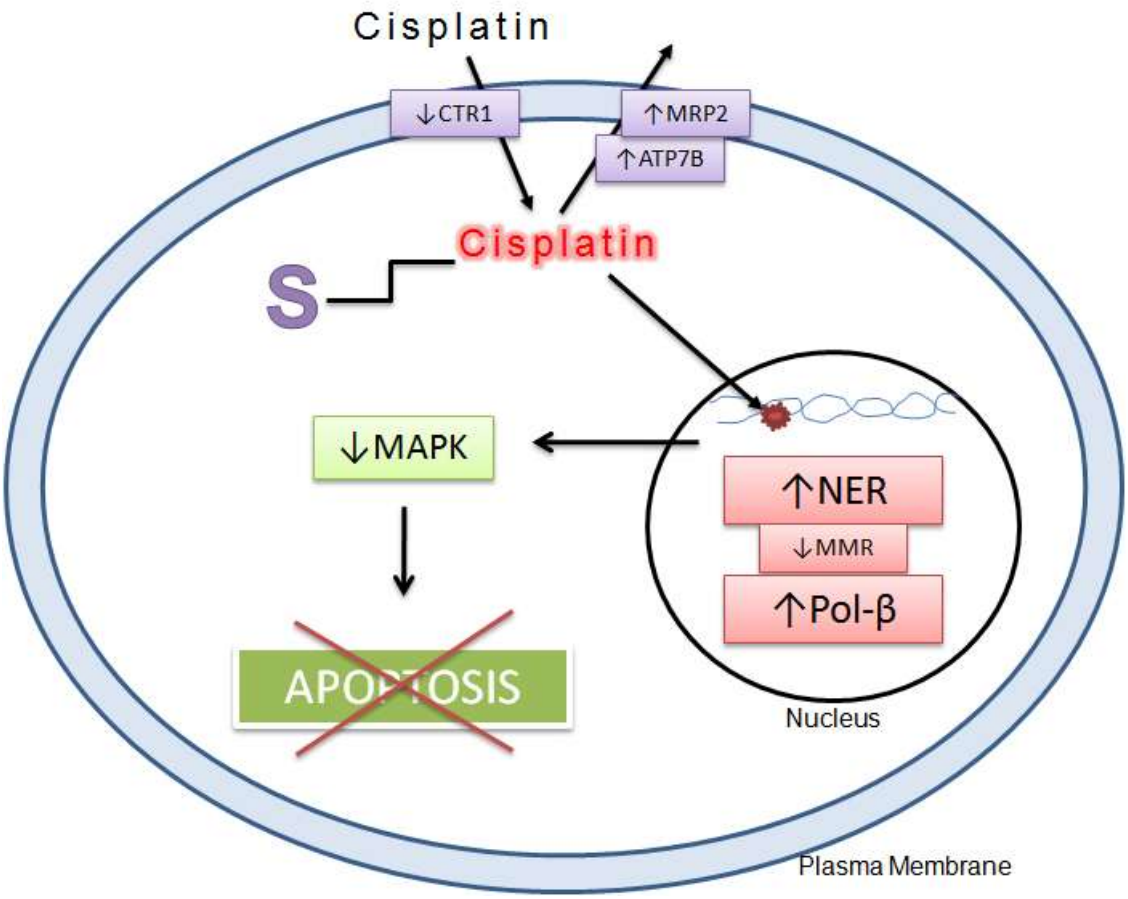
1.3. Mechanisms of cisplatin resistance

Acquired resistance to cisplatin is a major obstacle in the treatment of many cancers. Patients with solid tumours such as ovarian, testicular, head and neck, and small cell lung cancer are inherently sensitive to cisplatin as a first-line treatment (27). However, most patients who relapse acquire cisplatin resistance (27). For example, more than 70% of ovarian cancer patients initially respond to platinum therapy, but recurrence with resistant disease leads to a 25% five-year survival rate. The situation is more dire in small cell lung cancer where 95% of patients develop resistant tumours (27). Resistance to cisplatin occurs when cellular events interfere with the process of apoptosis. This is manifested through different mechanisms including reduced uptake and increased export of the drug, inactivation by thiol-containing species, increased DNA repair, and decreased apoptotic response (Figure 1.3) (1, 28).

1.3.1. Mechanisms of reduced cisplatin-DNA interactions

Resistance can be caused by a decrease in intracellular concentrations of cisplatin, acquired by reducing its uptake or by increasing its export from the cell. Cisplatin can enter the cell by two methods: by slow, passive diffusion and by membrane transporters (13). The membrane transporter copper transporter-1 (CTR1), is implicated in cisplatin uptake since its loss can lead to an increase in drug resistance (29, 30). In human ovarian cancer cell lines, cisplatin was shown to internalize and cause the degradation of CTR1, outlining a possible mechanism of resistance (30). Cisplatin can also be preferentially exported from the cell. Efflux protein copper-transporting P-type adenosine triphosphate (ATP7B), a copper exporter that shuttles between the Golgi and the plasma membrane, is overexpressed in cisplatin-resistant cells (31, 32) and its increased expression is correlated with poor survival in ovarian cancer patients (33). It uses the vesicular secretory pathway to sequester cisplatin into vesicles before exporting

Figure 1.3. Mechanisms of cisplatin resistance. Platinum resistance may occur from reduced uptake of cisplatin into the cell and increased export out of the cell (purple). This occurs by loss of the import transporter CTR1 and by overexpression of efflux proteins ATP7B and MRP2. Once cisplatin enters the cell, it can be inactivated by binding to sulphur-containing proteins. When cisplatin creates adducts with DNA, DNA repair mechanisms can be altered in cisplatin-resistant cells (red). An increased NER pathway repairs cisplatin adducts, a deregulated MMR pathway prevents apoptosis initiation through a p53-independent mechanism, and upregulated Pol-β causes an increased tolerance to damaged DNA through the replicative bypass pathway. Finally, a decrease in apoptotic response will render cells resistant to cisplatin treatment (green). This can be facilitated by a decrease in MAPK signals and a downregulation of components of the extrinsic and intrinsic apoptosis pathway.


Cisplatin
↓CTR1
↑MRP2
↑ATP7B
Cisplatin
S
↓MAPK
↑NER
↓MMR
↑Pol-β
APOPTOSIS
Nucleus
Plasma Membrane

it from the cell (14). Another transporter protein involved in resistance, multidrug resistance protein 2 (MRP2), is overexpressed in cisplatin resistant cells (34), can confer resistance when transfected into normal cells (35) and when inhibited can increase sensitivity to cisplatin (36).

Once the cisplatin molecule becomes aquated and activated within the cell, it can bind both DNA and proteins, with proteins representing 75-85% of bound cisplatin (17). Platinum has a high affinity for sulphur and can easily bind the sulphur-containing amino acids cysteine and methionine (13). Proteins rich in these amino acids such as glutathione and metallothionein can bind and inactivate cisplatin, thus impeding it from damaging DNA. High levels of these proteins have been correlated with cisplatin resistance both *in vitro* and *in vivo* (1, 13, 20). The detoxification of cisplatin can lead to increased efflux since the cisplatin-glutathione compound is preferentially exported by MRP2, therefore contributing to platinum drug resistance (37).

1.3.2. Alterations in DNA damage recognition and repair

Since the cytotoxic effect of cisplatin is due to DNA adduct formation and subsequent DNA damage, alleviating this damage by DNA repair mechanisms can lead to drug resistance (13, 20). The NER pathway is responsible for removing cisplatin adducts from DNA and has a major role in determining a cell's sensitivity to cisplatin. It is believed that having a low DNA repair capacity will augment the apoptotic response to cisplatin treatment (13). Testicular cancer cells, which are intrinsically hypersensitive to cisplatin with patient cure rates of up to 80%, are deficient in the NER proteins excision repair cross-complementing-1 (ERCC1), xeroderma pigmentosum complementation group A (XPA), and XP complementation group F (XPF) (38-40). ERCC1 functions by heterodimerizing with XPF to make 5' incisions in DNA at cisplatin adduct locations. Increased mRNA and protein levels of ERCC1 have been correlated with

resistance in ovarian cancer patients (13, 41, 42). Furthermore, inhibiting ERCC1 by antisense RNA in a resistant ovarian cell line enhanced its sensitivity to cisplatin *in vitro* (43).

The mismatch repair (MMR) pathway has a paradoxical role in cisplatin resistance. Its purpose is to correct base-base mismatches incurred after DNA replication (17). However, it is incapable of repairing cisplatin adducts and the successive attempt to repair the damaged DNA is believed to activate the apoptotic pathway through v-abl Abelson murine leukemia viral oncogene homologue (c-Abl) and p73 (14, 44). Thus, the MMR complex must be intact for cells to be sensitive to cisplatin and the downregulation or mutations of MMR genes have been correlated with reduced apoptosis and cisplatin resistance (20).

Another mechanism of cisplatin resistance is tolerance of DNA damage by the replicative bypass pathway, which allows DNA replication to occur prior to DNA repair. A correlation has been established between the extent of cisplatin resistance and DNA damage tolerance *in vitro* (45, 46). Tolerance is facilitated by the base excision repair enzyme DNA polymerase-β (Pol-β) that can replicate DNA in the presence of platinum adducts (17). Overexpression of Pol-β leads to increased cisplatin resistance in ovarian and breast tumour cells (47, 48).

1.3.3. Decrease in apoptotic response

Cytotoxicity caused by cisplatin is primarily due to apoptosis (13, 20). Cisplatin can induce apoptosis through the intrinsic and extrinsic apoptotic pathways (49), both of which when inhibited, can cause cisplatin resistance (50). The intrinsic pathway is initiated by intracellular signals, is mediated by the mitochondria and is controlled by Bcl-2 proteins (51). Pro- and anti-apoptotic signals converge on the outer mitochondrial membrane to disrupt the ratio of Bcl-2 and BAX, the most well known members of the Bcl-2 homology domain-containing family of proteins (17). BAX is a pro-apoptotic factor that oligomerizes in the outer mitochondrial

membrane to form pores through which cytochrome c is released. Bcl-2 inhibits BAX to maintain the integrity of the membrane and this balance determines whether the cell undergoes caspase 9-dependent apoptosis (17). Bcl-2 overexpression correlates with cisplatin resistance *in vitro* (52, 53) and, in a clinical setting, was associated with decreased response in ovarian cancer patients (54). A cDNA array revealed downregulation of BAX expression and an increase of Bcl-2 in a cisplatin-resistant prostate tumour cell line (55).

Extracellular ligands activate the extrinsic pathway by binding to transmembrane death receptors located on the cell surface (17). Once activated, the death receptor's intracellular death domain interacts with the Fas-associated death domain (FADD) protein to assemble into a death-inducing signalling complex (DISC). This complex activates procaspase 8 into its mature and active form, caspase 8, which then activates caspase 3 to start the cascade of protein cleavage responsible for the morphological changes characteristic of apoptosis (17). Caspase 3 is also activated by the intrinsic pathway and is known to be an amplifier of apoptotic signals (51). There are many examples of caspase deficiency leading to cisplatin resistance. Highly cisplatin-sensitive testicular cancer cells were made resistant by blocking the activation of the caspase 9 cell death pathway (56) and decreased caspase 3 and 8 activity correlated with resistance in an ovarian cancer cell line and a head and neck squamous cell carcinoma (HNSCC) cell line, respectively (57, 58).

Inhibitors of the apoptosis pathway provide a level of regulation that can be disrupted during cisplatin resistance. The X-linked inhibitor of apoptosis protein (XIAP) family of proteins block apoptosis by binding to and inhibiting the enzymatic activity of cleaved caspases (59). Downregulating XIAP increased cisplatin sensitivity in ovarian (60) and prostate cancer cells (61) while overexpression of XIAP in ovarian cancer cells increases cisplatin resistance

(62). The overexpression of another inhibitor of apoptosis protein, survivin, also correlates with cisplatin resistance in prostate, ovarian and gastric cancer cell lines (63-65).

The MAPK cascades, which are activated by stress signals and can lead to apoptosis, have also played key roles in cisplatin cytotoxicity and resistance (14, 28). Although the molecular events leading from cisplatin-induced DNA damage to MAPK activation remain to be elucidated, it is known that the downstream response depends on which family member is activated (2). Activation of p38 leads exclusively to pro-apoptotic outcomes, JNK activation has a mainly pro-apoptotic role, while ERK can induce both cell survival and cell death (2). There have been many examples of reduced activation of the p38 pathway leading to increased resistance to cisplatin (28, 66, 67) and, although controversial, inhibiting JNK and ERK have also been found to attenuate cisplatin-induced apoptosis in a number of cell line models (23, 24, 57, 68).

Resistance to cisplatin is a major roadblock in the successful treatment of cancer patients. Understanding the mechanisms of resistance will allow the development of therapies that target deregulated pathways, resulting in better drug efficacy.

1.4. Activating transcription Factor 3

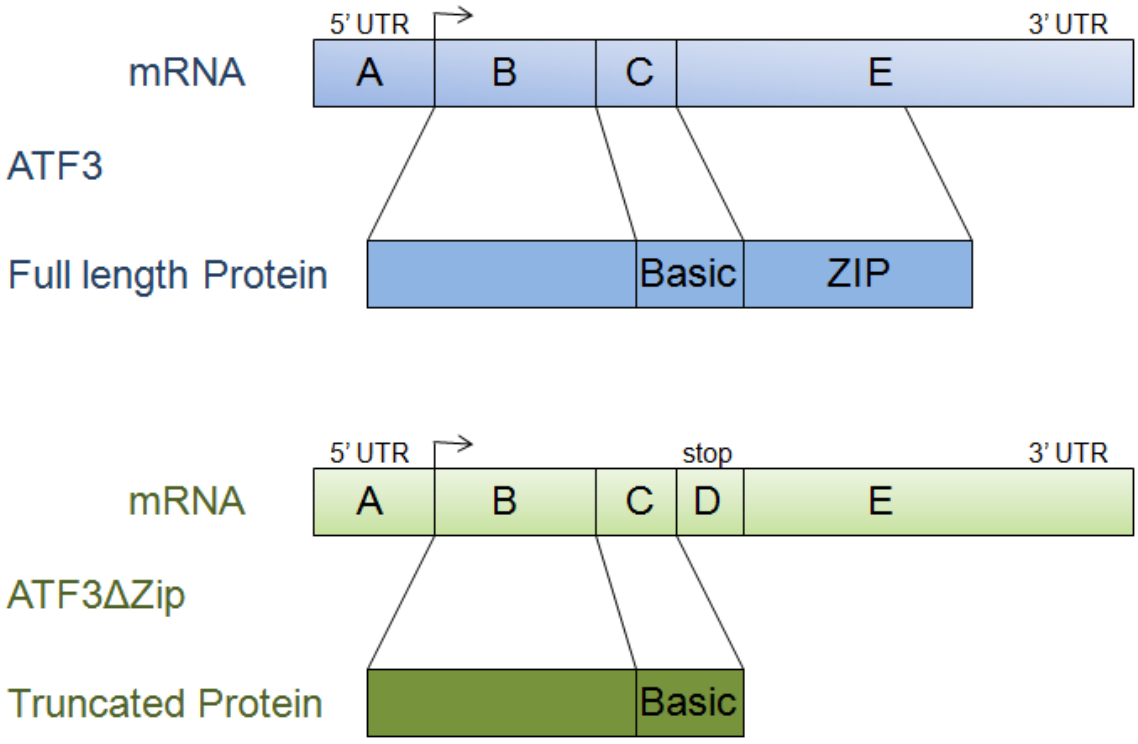
1.4.1. ATF3 gene

Activating transcription factor (ATF) 3 is a member of the ATF/cAMP responsive element binding (ATF/CREB) family of basic region-leucine zipper (bZip) transcription factors (69). Family members homodimerize and heterodimerize through their bZIP regions and bind DNA at ATF/cAMP responsive element (ATF/CRE) consensus sequences (TGACGTCA) (70, 71). The *ATF3* gene is located on chromosome 1q, is 181 amino acids in length and translates

into a 21 kDa protein (71). It has four exons (A, B, C, E) spread over 15 kilobases and it is transcribed into two splice variants (72). The full-length transcript consists of exon A, which encodes the 5'-untranslated region (UTR); exon B contains the start codon and the N-terminus; exon C is the basic amino acid region responsible for DNA binding; and exon E is the leucine zipper (ZIP) dimerization domain and the 3'-UTR (Figure 1.4) (71). The alternatively spliced variant, ATF3ΔZip (14 kDa), has an additional exon (exon D) that contains a premature stop codon before the ZIP dimerization domain. This truncates the protein after exon C, preventing it from dimerizing and properly binding DNA at ATF/CRE consensus sites (72). Although the regulation of *ATF3*'s alternative splicing remains to be discovered, there are clues towards the splice variants' distinct function. Several studies have observed that truncated ATF3 acts as a transcriptional activator, possibly by sequestering transcriptional co-repressors (72-74). Another study found that the ATF3 splice variant represses NFκB-dependent transcription by displacing a positive cofactor (75).

There are several transcription factor binding sites located within the *ATF3* gene promoter. ATF/CRE, AP-1 and NF-κB are inducible sites, which are known to be activated upon stress signals, and the Myc/Max, E2F, and p53 binding sites are involved in cell cycle regulation (71, 76). The expression of ATF3 mRNA is transient due to its ability to repress its own promoter (77). Both the ATF/CRE site and a novel "-20" site downstream of the TATA box must be bound by ATF3 for efficient auto repression (77). There is a lack of evidence for posttranslational modifications of the ATF3 protein, although there are many potential modification sites. These include several serine, threonine, tyrosine and lysine residues, which compose ~20% of the molecule (78).

Figure 1.4. ATF3 mRNA isoforms. ATF3 mRNA contains exons A, B, C, E to form a full-length protein including a basic DNA binding region and a ZIP dimerization domain. ATF3ΔZip mRNA contains exons A, B, C, D, E where exon D encodes a stop codon, thus truncating the protein after the basic DNA binding region. The ATF3ΔZip protein cannot dimerize with other proteins and cannot properly bind DNA. Adapted and modified from Liang 1996 (71).


5' UTR
3' UTR
mRNA
A
B
C
E
ATF3
Full length Protein
Basic
ZIP
5' UTR
stop
3' UTR
mRNA
A
B
C
D
E
ATF3ΔZip
Truncated Protein
Basic

An alternate promoter of the *ATF3* gene was recently identified as P1 (79). It is located 43.5 kb upstream of the currently known promoter, P2. P1 contains similar transcription factor binding sites to P2 including ATF/CRE, AP-1, p53, E2F, and NF-κB. Both P1 and P2 contributed to the induction of *ATF3* gene transcription by exposure to doxorubicin, hydrogen peroxide, and thapsigargin (79). Although both promoters produce identical ATF3 transcripts, their 5'UTR differs and results in differential mRNA translation efficiencies. In the LNCaP human prostate cancer cells the P1 promoter is constitutively active, which can account for the high level of basal ATF3 expression observed in that cell line (79).

ATF3 is also a member of the larger AP-1 DNA binding protein group composed of the bZIP transcription factors Jun (c-Jun, JunB, JunD), Fos (C-Fos, FosB, Fra-1, Fra-2), and ATF (ATF2, ATF3, B-ATF) (80). ATF3 is known to homodimerize with itself and to form heterodimers with ATF2 and Jun protein members (69, 70). In general, the ATF3 homodimer is considered a repressor of target genes, but when partnered with other transcription factors it can both activate and repress transcription (69). For example, ATF3/c-Jun and ATF3/JunD activate promoters with ATF/CRE binding sites, whereas the ATF3/JunB heterodimer activates genes with CRE-containing promoters but represses genes with AP-1-containing promoters (81). Thus, ATF3's role in transcription cannot be generalized. Evidence suggests that ATF3 may repress or activate the transcription of target genes depending on its dimerizing partner and the promoter context (72).

1.4.2. ATF3 as a stress-inducible gene

ATF3 was first linked to the cellular stress response by Chen *et al.* in 1996 when they observed that ATF3 mRNA expression was induced by physiological stress injuries such as regenerating and damaged liver, damaged heart and brain seizures (82). Hai *et al.* later termed

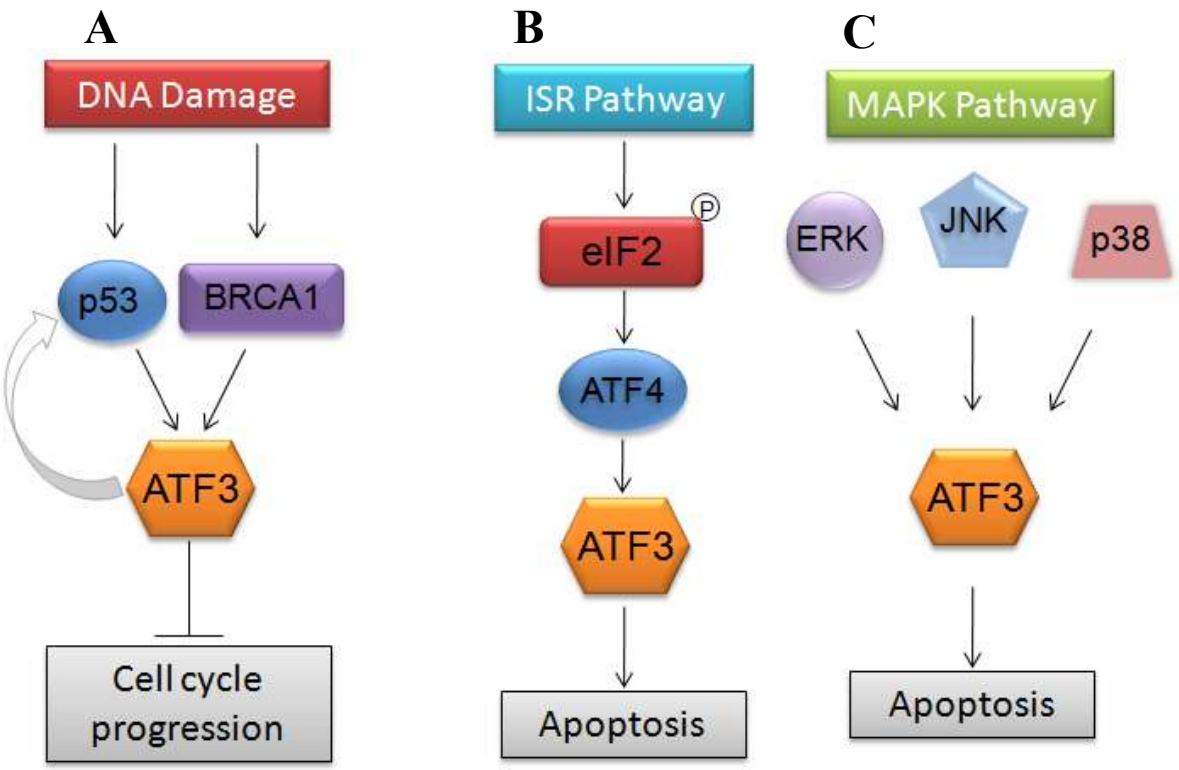
ATF3 a stress-inducible gene after noting that its mRNA level is low or non-detectable in most cells, but is greatly increased upon the exposure of cells to stress signals (83). ATF3 induction is not tissue or stress specific; it is a common cellular response to many different stress signals. In cell culture, ATF3 is induced by cytokines (84, 85), genotoxic agents such as ultraviolet light (UV), ionizing radiation (IR), methyl methanesulphonate (MMS) and cisplatin (86-89), and inducers of the JNK pathway such as anisomycin (90), cyclohexamide (91), and doxorubicin (92). The mitotic inhibitors taxol and colchicines (93) and the proteasome inhibitors lactacystin and MG132 (94) have also been reported to induce ATF3 expression. As well, the oxidative stressor hydrogen peroxide (95) and the ER stressor thapsigargin (96) are known ATF3-inducing agents.

However, *ATF3* has recently been characterized as an "adaptive response" gene because it participates in various cellular processes to adapt to extra- and/or intracellular changes (90). Examples of this include the induction of ATF3 expression by adipokines, secreted factors of adipocytes, in MCF7 cells (97) and up-regulation during S-phase (98, 99).

1.4.3. Mechanisms of ATF3 induction

Depending on the signal and cell type, the mechanism of ATF3 induction can involve many different cellular pathways (70). Three pathways have emerged as common mechanisms of stress-induced ATF3 expression, namely the DNA damage response, the integrated stress response (ISR) pathway, and the MAPK pathway (Figure 1.5). As discussed previously, DNA damage triggers the activation of genes and proteins involved in cell cycle arrest, DNA repair and programmed cell death (Figure 1.2). Both the p53 and BRCA1 DNA damage response pathways can regulate ATF3 induction (Figure 1.5A). ATF3 was first identified to be a direct target of p53 by a microarray approach in a human lung cancer cell line (100). This finding was

Figure 1.5. Mechanisms of ATF3 induction. **A**, DNA damage induces the expression of p53 and BRCA1, both of which activate ATF3. The downstream effect of DNA damage-induced ATF3 is often to halt progression of the cell cycle. ATF3 can positively regulate p53 through a positive feedback loop. **B**, The ISR pathway converges on eIF2 to phosphorylate it. This activates ATF4 and ATF3, leading to the initiation of apoptosis. **C**, The three members of the MAPK pathway, ERK, JNK, and p38 can all lead to ATF3 activation through their respective signalling pathways. Often, these signals result in the initiation of apoptosis.


A
DNA Damage
p53
BRCA1
ATF3
Cell cycle progression
B
ISR Pathway
eIF2
P
ATF4
ATF3
Apoptosis
C
MAPK Pathway
ERK
JNK
p38
ATF3
Apoptosis

confirmed by a study that used UV and MG132 to upregulate ATF3 protein expression in a p53-dependent manner (76). This study also located two p53-responsive elements in the *ATF3* gene promoter. ATF3's transcriptional role upon p53 activation was recently discovered to be the direct inhibition of the *Cdc25a* gene, thus contributing to cell cycle arrest (101). Interestingly, a positive feedback loop has been suggested where the ATF3 protein binds p53 to prevent its ubiquitination and degradation (89). Therefore, sustained ATF3 levels in the cell might further halt the cell cycle through continued *Cdc25a* transcriptional inhibition. ATF3 is also a direct target of BRCA1. Harkin *et al.* used a microarray approach and Fan *et al.* used a reporter construct to show that the *ATF3* gene can be transcriptionally activated by BRCA1 (102, 103). Overexpression of ATF3 by the Tet-on system in HeLa cells slowed transition from G1 to S phase, thus slowing down cell cycle progression (102, 104). Taken together, ATF3 induction downstream of DNA damage is involved in the tumour suppressor function of halting the cell cycle.

ATF3 also plays a central role downstream of the ISR pathway (Figure 1.5B). This stress response pathway is activated upon viral infection, oxidative stress, ER stress and nutritional stress (105). These stress signals converge on the eukaryotic initiation factor 2 (eIF2) kinase and activate it by phosphorylation, which leads to ATF4 activation and subsequent ATF3 transcription. In this pathway, ATF3 is upstream of the pro-apoptotic factor C/EBP homologous protein (CHOP), also known as growth arrest DNA damage inducible gene 153 (GADD153) (106). Several agents have been shown to induce ATF3 expression through the ISR pathway such as lovastatin (107), thapsigargin (105) and the histone deacetylase (HDAC) inhibitor M344 (108).

A number of different stimuli induce ATF3 through the MAPK pathway (Figure 1.5C). Anisomycin, a stress inducer, upregulates ATF3 through the p38 pathway by binding of the ATF2/c-Jun heterodimer to *ATF3*'s gene promoter, leading to caspase 3 cleavage and apoptotic outcomes (71, 90, 109). In a cardiovascular disease model, elevated levels of homocysteine induced ATF3 through the JNK pathway by ATF2/c-Jun binding to the ATF/CRE site on the *ATF3* gene promoter (110). UV irradiation induced apoptosis by activating both the p38 and JNK pathways, which converged on ATF3, a necessary component of the cell death pathway in this study (111). Tolfenamic acid, a non-steroidal anti-inflammatory drug, was shown to induce all three MAPK pathways (p38, JNK and ERK) which phosphorylated ATF2 and led to ATF3-mediated apoptosis (112). Cisplatin was also shown to activate ATF3 through all three MAPK pathways and ATF3 was important for cisplatin's cytotoxicity (113).

1.4.4. ATF3 downstream targets

ATF3 induction by genotoxic agents can lead to the activation and repression of proteins involved in cell cycle regulation and apoptotic pathways. As well, there is an emerging regulatory role for ATF3 in immunity and the inflammatory response (Table 1). As a cell cycle regulator, ATF3 can directly bind the p53 protein to prevent its ubiquitination and degradation, and thus augment its function (89). Upon treatment with the mitotic inhibitor paclitaxel, ATF3 can also stabilize p73 through the same mechanism as above (114). Therefore, ATF3 is supporting the tumour suppressive roles of p53 and p73, which cause cell cycle arrest and apoptosis (89, 114). Cyclin D1 is another regulator of cell proliferation that can be under the control of ATF3. Mitogenic signals allow cyclin D1's interaction with cyclin-dependent kinase (CDK)-4 and/or CDK6, which leads to activation of the E2F transcription factor and subsequent

Table 1. ATF3 downstream targets

Target	ATF3 function	Reference
Cell Cycle Regulation		
P53	Protein interaction prevents degradation	(89)
P73	Protein interaction prevents degradation	(114)
Cyclin D1	Transcriptional repressor	(87)
Id-1	Transcriptional repressor	(116-118)
Apoptotic Pathway		
CHOP	Transcriptional activator	(106)
	Transcriptional repressor	(86, 119)
Immune System and Inflammatory Response		
IL-6, IL-12b	Transcriptional repressor	(120, 121)
IL-4, IL-5, IL-13, CCL4	Transcriptional repressor	(122)

cell cycle progression (115). ATF3 can directly target the *cyclin D1* gene promoter, repressing its expression and subsequently leading to cell cycle arrest at the G1-S checkpoint (87). Another ATF3 target involved in the cell cycle is inhibitor of differentiation (Id-1), an oncogene involved in cell growth and invasion (116). ATF3 has consistently been shown to repress the *Id-1* gene promoter in several *in vitro* models, supporting ATF3's role as a transcriptional regulator of cell cycle control genes (116-118).

CHOP is a known inducer of apoptosis and functions by downregulating Bcl-2, a promoter of cell survival (123). As discussed earlier, inducers of the ISR pathway upregulate CHOP in an ATF3-dependent manner (105, 106). As well, activation of the p38 MAPK pathway is known to induce CHOP expression (2). However, cellular stressors such as arsenite and carbon tetrachloride are shown to downregulate CHOP expression through direct ATF3 repression of the *CHOP* gene promoter (86, 119). Chen *et al.* revealed a negative feedback loop where unstressed liver cells expressing high levels of CHOP negatively regulate ATF3 function (82). This is done by forming a non-functional heterodimer through their bZIP domains and preventing ATF3 from acting on target promoters (82). The ability of ATF3 to activate and repress CHOP transcription can help explain ATF3's pro- and anti-apoptotic roles upon cellular stress.

Gilchrist *et al.* were the first group to link ATF3 with the innate immune system, a first line of defence against infection (120). A DNA microarray coupled with systems biology analysis revealed that ATF3 is induced upon TLR-4 activation (120). TLR-4 activates the interleukin (IL)-6 and IL-12b cytokines through NF-κB and ATF3 was found to repress the cytokine signal shortly after it was initiated (120). The same study also found that ATF3 repressed 11 genes involved in macrophage signalling, thus highlighting ATF3's role in a

negative feedback loop of the innate immune system. This new function was validated by another study that found ATF3 to be induced by a wide range of TLRs (TLR-2/6, -3, -5, -7, -9) located on the cell surface membrane and in intracellular endosomes (124).

ATF3 also has an important role in a negative feedback loop of the inflammatory response. Its role is to prevent acute inflammatory syndromes caused by elevated cytokine production (125). ATF3 is induced in an asthma mouse model and *atf3*-null mice had more severe asthmatic symptoms than wild type mice (126). ATF3 appeared to elicit its inflammatory suppression by directly antagonizing the transcription of pro-inflammatory cytokines (IL-4, IL-5, IL-13) (126) and a chemokine (chemokine C-C motif ligand 4, CCL4) (122). Interestingly, ATF3 appears to be repressed in patients with severe asthma compared to patients with mild disease (127).

1.5. ATF3's role in cancer

ATF3 is described to have a dichotomous role in cancer progression (128). It can act either as a tumour suppressor to induce cell cycle arrest and apoptosis or as an oncogene to promote cell survival and proliferation. It is proposed that the function of ATF3 is context-dependent and that its activity varies based on available cellular binding partners and the status of malignancy (87, 129). The concept of a dichotomous role in cancer development has been shown for molecules such as transforming growth factor (TGF)-β (128). TGF-β expression was shown to induce apoptosis or cell cycle arrest in normal cells but promote metastasis in advanced tumours (130-132). Interestingly, TGF-β can induce ATF3, further supporting a dichotomous role for ATF3 in cancer development and progression (117). A powerful example of ATF3's ability to function differentially under varying degrees of malignancy came from the Yin group

in 2008. They discovered that under stress, ATF3 promotes apoptosis in normal MCF10A mammary epithelial cells but protects aggressive MCF10CA cancer cells by inhibiting cell cycle arrest and enhancing cell migration and invasion (128). Another explanation of ATF3's dichotomous role is that it activates p53, which can initiate cell death or survival (through cell cycle arrest) depending on stress input and cell type (129).

Many studies have described a role for ATF3 in varying cancer models. Several have focused on ATF3's role as a tumour suppressor and its ability to induce cell cycle arrest and apoptosis. Lu *et al.* described that ATF3 can directly repress cyclin D1 by binding to an ATF/CRE site on the *cyclin D1* gene promoter, leading to cell cycle arrest at the G1-S checkpoint (87). In the same study, ATF3 was also able to suppress Ras-stimulated tumourigenesis both *in vitro* and *in vivo*. Overexpression of ATF3 in a fibrosarcoma cell line was shown to accelerate caspase protease activation and apoptosis, and ectopic expression of ATF3 in ovarian cancer cell lines suppressed growth and enhanced caspase-3 mediated apoptosis (133, 134). In a HeLa cell inducible system, high protein levels of ATF3 reduced colony formation by half (103). Furthermore, overexpression of ATF3 suppressed colon tumour formation *in vivo* and reduced cell invasion and migration in a colon cancer cell line (135, 136).

In contrast, many groups explored ATF3's role as an oncogene and its ability to induce cell survival and proliferation. Ishiguro and colleagues discovered that ATF3 is expressed in the highly metastatic B16 melanoma subline, but not in the parental B16 cells (137). Furthermore, expression of ATF3 in the low metastatic parental line converted them into highly metastatic cells. The same group later showed that knocking down ATF3 was protective against tumourigenicity in HT29 colon cancer cells (138). As well, ATF3 transfection increased the invasiveness of prostate cells *in vitro*, and mice injected with prostate cells stably expressing

ATF3 showed significantly more lung metastasis than the control (139). In a breast cancer model, ATF3 plays an integral role in promoting cell motility and epithelial-mesenchymal transition (EMT) in developing mammary carcinomas in transgenic mice (140, 141). ATF3's controversial role in cancer development and progression warrants further study to characterize the circumstances of ATF3's oncogenic and tumour suppressive roles.

1.6. Proteasome inhibition as a cancer therapy

The ubiquitin-proteasome system (UPS) consists of an energy-dependent protease that degrades polyubiquitinated proteins to maintain cellular homeostasis (142). It is a multi-subunit complex that consists of a 19S regulatory cap, which de-ubiquitinates and denatures target proteins, and a barrel-shaped 20S core subunit that contains the catalytic cleavage activity (143). The proteasome has many different substrates including proteins involved in cell cycle regulation, apoptosis and cellular stress response (144). Proteasome inhibitors have been demonstrated to selectively kill cancer cells (143, 144). Although the explanation for this has not been mechanistically solved, it is postulated that cancer cells rely heavily on the proteasome for proper cell division and cell metabolism, and increased protein synthesis prior to mitosis imposes an extra strain on the UPS (143-145). Bortozemib (also known as PS-341 and Velcade) is the first proteasome inhibitor to be approved for treatment of multiple myeloma and mantle cell lymphoma, and acts by inhibiting the catalytic activity of the 20S proteasome (146).

The mechanism of proteasome-induced apoptosis is currently under study and involves many different aspects of cell regulation. NF-κB, a pro-survival transcription factor, is constitutively active in a large proportion of cancers but becomes inactivated after proteasome inhibition (143). This is because NF-κB activity requires the proteasome to degrade its inhibitor,

IκB. Proteasome inhibition also stabilizes the pro-apoptotic proteins p53, Bim, Noxa, and Bik, thus enabling the apoptotic response pathway (143). Furthermore, inhibiting the UPS causes an accumulation of misfolded proteins in the cell, leading to the generation of reactive oxygen species (ROS) and ER stress (143). Upon ER stress, the cell launches the unfolded protein response (UPR) to recover from insult and restore proper ER homeostasis (147). This is manifested through the upregulation of protein chaperones, which are capable of enhancing protein folding, and ER-associated protein degradation (ERAD) proteins, which increase the degradation of misfolded and aggregated protein substrates (147). The MAPK stress response cascades, specifically the JNK and p38 pathways, are also initiated (147). However, if the ER stimulus is excessive, such as the case with proteasome inhibition, the pro-survival defences are overloaded and pro-apoptotic components such as CHOP and caspase 4 are upregulated to induce cell death (147-150).

The crucial link between proteasome inhibitor-induced UPR response and apoptosis has been shown to be the ISR pathway (148, 151-153). Proteasome inhibition by bortezomib activates PKR-like ER kinase (PERK), an ER transmembrane serine/threonine kinase, which is a member of the ISR pathway and responsible for ER signalling (105, 147, 152). The mechanism of PERK activation involves the ER chaperone 78-kDa glucose-regulated protein (GRP78) also known as the immunoglobin heavy-chain-binding protein (BiP), which releases PERK upon the accumulation of misfolded proteins, allowing it to homodimerize and autophosphorylate (154). Another proteasome inhibitor, MG132, was shown to activate general control non-derepressible-2 (GCN2) and heme-regulated inhibitor (HRI), both protein kinase members of the ISR pathway (105, 151, 155). Activation of these ISR pathway kinases leads to the phosphorylation of eIF2α, which shuts down global protein translation (105). ATF4 is preferentially translated under eIF2α

phosphorylation due to the ribosome bypassing an inhibitory element in *ATF4*'s 5'-UTR (105). The ATF4 transcription factor then activates ATF3, which upregulates CHOP protein expression, leading to the initiation of apoptosis (105).

Proteasome inhibitors have been shown to synergize with ER stressors such as celecoxib and cisplatin in a wide range of cancer cell models (148, 149, 156-158). There are also many clinical trials investigating the combination of proteasome inhibitors and chemotherapy regimens in solid tumours (159). In a phase II study, bortezomib in combination with gemcitabine and carboplatin increased survival in patients with advanced NSCLC (160). Therefore, proteasome inhibitors show promise as single-agents and in combination with chemotherapeutics in a variety of cancer types.

2.0. Rationale and Hypothesis

Resistance to cisplatin is a major limitation for the treatment of many cancers. Identifying the mechanisms regulating cisplatin-induced apoptosis will lead to the discovery of novel therapeutic approaches. ATF3 induction by cisplatin was recently shown to act through the MAPK pathway, the suppression of which is implicated in cisplatin resistance (28, 113). Furthermore, targeting ATF3 by shRNA attenuated the cytotoxic effect of cisplatin in a lung cancer cell model (113). Therefore, combining cisplatin treatment with an inducer of ATF3 has the potential to sensitize cells to its cytotoxic effects. There is evidence for increased cytotoxicity with the histone deacetylase M344 (108). Treatment of a lung cancer cell line with M344 enhanced cisplatin-induced cytotoxicity (108). ATF3 was shown to play a role in this enhanced cytotoxicity since knockdown of ATF3 by shRNA attenuated the increased sensitivity from the addition of M344 (108). Taken together, ATF3 can regulate cisplatin's ability to kill

cancer cells and inducing its expression may overcome drug resistance. Further characterization of ATF3's role in cisplatin cytotoxicity will lead to novel therapeutic approaches that could improve the efficacy of this important class of chemotherapies.

Hypothesis

ATF3 plays a critical role in regulating tumour cells' toxic response to cisplatin.

2.0.1. Objectives

1. Delineate the mechanism by which ATF3 regulates cisplatin-induced cytotoxicity
2. Potentiate cisplatin cytotoxicity by enhancing ATF3 expression
3. Evaluate the predictive value of ATF3 expression in patient tumour samples

Chapter 2: Materials and Methods

2.1. Tissue Culture

The WI38, A549, PC3, and MCF7 cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). Cell lines SKOV3 and A2780s were kindly provided by Dr. Barbara Vanderhyden, (Ottawa Hospital Research Institute (OHRI), Ottawa, ON, CAN). All cell lines were maintained in Dulbecco's-MEM (Media Services, Ottawa Hospital Regional Cancer Centre, Ottawa, ON, CAN) supplemented with 10% fetal bovine serum (Wisent Inc., St-Bruno, QC, CAN) and 100 μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). Unless otherwise described, cells were treated for 24 hr with cisplatin (provided by the pharmacy at the Ottawa Hospital Regional Cancer Centre) alone or in combination with a 24 hr pre-treatment of disulfiram (10 mM stock diluted in DMSO, Sigma-Aldrich, St-Louis, MO, USA).

2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

In a 96-well flat-bottomed plate (Corning Costar #3595, Corning, NY, USA) 4,500 cells/150 µL of cell suspension were used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. Cells were treated with cisplatin alone and incubated for 48 hr at 37°C, or were treated with disulfiram for 24 hr followed by treatment with cisplatin for 48 hr. Following treatment, 42 µL of a 5 mg/mL solution of the MTT tetrazolium substrate (Sigma-Aldrich) in phosphate buffered saline (PBS) was added to each well and incubated for 4 hr at 37°C. The resulting violet formazan precipitate was solubilised by the addition of 82 µL of a 0.01 mol/L HCl/10% SDS (Sigma-Aldrich) solution, and allowed to incubate further at 37°C for 24 - 72 hr. The plates were analyzed on a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader using Gen5 software, both from Biotek Instruments (Winooski, VT, USA), at 570 nm to determine the absorbance of the samples.

2.3. Western Blotting

Protein samples were collected in RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS) containing 1x Protease Inhibitor Cocktail (Sigma-Aldrich) and protein content was quantified using a commercially available protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL, USA) and a Biomate3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were separated on 8 - 12% SDS polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA, USA). Blocking was performed with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). For all subsequent immunoblotting, antibodies were diluted to the appropriate concentration in 5% milk in TBS-T. Blots were incubated with the following

primary antibodies for 1 hr at room temperature or overnight at 4ºC: rabbit-anti ATF3 (1:1000, C-19, Santa Cruz, Santa Cruz, CA, USA), rabbit-anti PARP (1:1000, Cell Signaling Technology, Lake Placid, NY, USA), mouse-anti cyclin D1 (1:1000, A-12, Santa Cruz), rabbit-anti CHOP (GADD153) (1:1000, R-20, Santa Cruz), and mouse-anti actin (1:10000, Sigma-Aldrich). Following 3 washes in TBS-T, blots were incubated with the appropriate horseradish peroxidase (HRP)-labelled secondary antibody (goat-anti-rabbit-HRP, goat-anti-mouse-HRP, 1:5000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 hr at room temperature in 5% milk in TBS-T. The chemiluminescent substrate used was Supersignal West Pico (Pierce) and the visualization of the protein bands was performed using the GeneSnap image acquisition system (Syngene, Frederick, MD, USA).

2.4. Chromatin Immunoprecipitation (ChIP) Assay

Cells treated for 24 hr in 10 cm dishes were fixed in 1% formaldehyde (BDH, VWR International, Mississauga, ON, CAN) for 20 min at room temperature in order to cross-link the DNA and protein. Fixation was stopped by quenching with 2.5 mM glycine solution to a final concentration of 200 mM for 5 min. Cells were then washed twice with ice-cold PBS and harvested in 1 ml cold PBS by centrifugation at 4ºC for 5 min at 5,000 rpm. The pellet was resuspended in 90 µL lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) supplemented with 1x Protease Inhibitor Cocktail, 1 mM 1,4-dithio-DL-threitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (all Sigma-Aldrich). The lysates were sonicated on ice using a Sonicator 3000 (Misonix, Farmingdale, NY, USA) at power setting #1 for a total of 3 min (10 sec on/off pulses) to shear DNA to an average size of 300 to 1000 base pairs. Sonicated

lysates were cleared of debris by centrifugation at 4ºC for 15 min at 14,000 rpm. Input controls were removed from each sample and stored at -20ºC.

Sonicated lysates were divided into negative controls and samples, then diluted 10-fold with dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100), supplemented with 1x Protease Inhibitor Cocktail, 1 mM DTT and 1 mM PMSF (all Sigma-Aldrich). Positive sample cell lysates were immunoprecipitated by overnight rotation at 4ºC with rabbit-anti ATF3 (1:200, Santa Cruz) primary antibody. Negative controls were incubated overnight with rotation at 4ºC in the absence of primary antibody. Immune complexes were collected by 2 hr rotation at 4ºC with the addition of 40 µL of protein A agarose/salmon sperm DNA 50% slurry (Millipore) to both positive samples and negative controls. The agarose beads/immune complexes were pelleted gently by centrifugation at 4ºC for 1 min at 3,000 rpm. The beads were washed with 1 ml of the following buffers by rotation for 10 min at 4ºC, then pelleted gently by centrifugation at 4ºC for 1 min at 3,000 rpm, discarding the supernatant following each wash: Buffer A (low salt; 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 150 mM NaCl) once, Buffer B (high salt; 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 500 mM NaCl) once, Buffer C (1% NP-40, 1% sodium deoxycholate, 20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.25 M LiCl) once and TE washing buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) twice. All antibody complexes were eluted with a total volume of 400 µL elution buffer (freshly prepared 1% SDS, 100 mM $NaCHO_3$) by rotating at room temperature for 30 min. The agarose beads were removed from the samples by centrifugation for 1 min at 3,000 rpm. The DNA-protein cross-links were reversed by overnight incubation with 100 µg proteinase K (Roche Diagnostics, Laval, QC, CAN) at 65ºC.

DNA was purified using a QiaQuick PCR Purification Kit (Qiagen, Toronto, ON, CAN) according to the manufacturer's instructions. Purified DNA was eluted in 50 µl ddH_2O and samples were stored at -80ºC. Quantitative PCR was performed using a Roche LightCycler Version 3 (Roche Diagnostics) with the following amplification conditions: 95ºC for 1 min, 40 PCR cycles of 95ºC for 1 sec, 58ºC for 10 sec, 72ºC for 5 sec. The binding of ATF3 to the *CHOP* gene promoter region was determined using the following primer pair: forward 5-GGCGGGACCCCAAAACCTACC-3' and reverse 5'-GCTCCTGAGTGGCGGATGCG-3'. PCR products were resolved on 1.6% agarose gels.

2.5. High throughput chemical library screen

A panel of cancer cell lines (A549, PC3, A2780cp, SCC-25) were treated with a chemical library of 1200 FDA-approved compounds (Prestwick Chemical, Illkirch, FRA). The small molecule compounds have already undergone bioavailability and toxicity studies and have shown activity in humans (161). All compounds were supplied in a 10 mM stock diluted in DMSO and were used at a final concentration of 1 µM. The cell lines were exposed to the drug library alone for 72 hr, or were pre-treated with the drug library for 24 hr followed by cisplatin treatment (2 – 4 µg/ml) for 48 hr. The MTT assay was used to determine cell viability as described above (section 2.2). CalcuSyn (Biosoft, Cambridge, GBR), a dose-effect analyzing software for single and multiple drugs, was used to determine synergistic relationships between cisplatin and other compounds.

2.6. RNA isolation and RT-PCR

The use of ovarian cancer patient tumours for this restropective study was approved by the Ottawa Hospital Research Ethics Board (see Appendix I). Flash frozen tumours of predominantly serous histology were obtained from 49 patients who had received adjuvant, platinum-based chemotherapy. Patients gave informed consent for their tumour to be stored in the Ovarian Cancer Tissue Bank at the OHRI for the purpose of research. Per patient sample, total RNA was extracted from 3 separate 30 mg frozen tumour pieces using the RNeasy® kit (Qiagen). RNA concentrations were quantified using a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). One microgram of total RNA was reverse-transcribed to complementary DNA using Superscript II reverse transcriptase and oligo (dT) primer according to the manufacturer's protocol (Invitrogen). Quantitative, real-time polymerase chain reaction (RT-PCR) was performed with the Applied Biosystems ABI 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA). A quantitative RT-PCR reaction was carried out in a total volume of 25 µl that contained 2.5 µl of synthesized cDNA, 1.25 µl of TaqMan Gene Expression Assay Primer/Probe (20x) (Applied Biosystems, ATF3, HS00231069), 12.5 µl of TaqMan Universal PCR Master Mix (2x) (Applied Biosystems, 4304437) and 8.75 µl of RNase-free water for ATF3 expression. The endogenous control for ATF3 was the housekeeping gene, human GAPDH (20x) (Applied Biosystems, HS4333764-F). The positive control was A2780s treated with 2 µg/ml cisplatin for 24 hr. Amplification conditions were 95^{o}C for 5 min, 40 PCR cycles at 95^{o}C for 15 sec and 60^{o}C for 1 min. Gene expression per patient sample was determined by performing quantitative RT-PCR on three individual tumour pieces and expressing the data as the mean ± standard deviation (SD).

2.7. Immunohistochemistry (IHC)

Tissue microarrays (TMAs) were purchased from US Biomax (OV1501, OV482, LUC1501, LC482) with tumour grade and stage data available (US Biomax, Rockville, MD, USA). MCF7 and PC3 cells grown to 50% confluency on 2 x 15 cm plates each, were either treated with cisplatin (8 μg/ml) for 24 hr, or were untreated as controls. Cells were washed twice in PBS, harvested in 10 ml PBS/plate and combined with 20 ml of 20% Neutral Buffered Formalin (Sigma-Aldrich) for a final concentration of 10% Neutral Buffered Formalin. Cells were fixed at 4^{o}C for 1 hr, spun down at 1600 rpm for 10 min at 4^{o} C and then washed once in PBS. Formalin-fixed cells were paraffin-embedded, cut into 5 μm sections and allowed to dry at room temperature overnight. Slides were deparaffinised by washing in toluene for 3 times 5 min followed by washing in absolute alcohol for 2 times 1 min. Sections were washed in running distilled water for 5 min followed by a wash in TBS and loaded on the intelliPAT FLX automated slide stainer (Biocare Medical, Concord, CA, USA). Sections were incubated with 3% hydrogen peroxide in TBS for 10 min, rinsed in TBS for 5 min and blocked with universal blocking agent Background Sniper (Biocare Medical) for 20 min at room temperature. Staining was performed with an ATF3 antibody (1:200 dilution in DaVinci universal diluents, Biocare Medical) for 1 hr at room temperature. The slides were subsequently rinsed with TBS for 5 min, incubated with universal rabbit probe (Mach 4 universal polymer detection kit, Biocare Medical) for 5 min, and rinsed with TBS for 5 min before incubating with Rabbit HRP Polymere (Mach 4 universal polymer detection kit, Biocare Medical) for 10 min at room temperature. Finally, the slides were rinsed with TBS, developed for 5 min with DAB RTU (Betazoid DAB chromatogen kit, Biocare Medical) and then rinsed with water. Slides were counterstained in hematoxylin for 1 min, washed in running water, dipped in 0.2% HCl in 70% alcohol 5 times, washed in running

water 1 min, dipped once in 2% aqueous saturated lithium carbonate, washed in running water 5 min, dehydrated in absolute alcohol, cleared in toluene and mounted on cover slips with permount.

2.8. Evaluation of therapeutic interactions

The combination effect of disulfiram and cisplatin was evaluated by the Chou-Talalay method (162) using CalcuSyn computer software (Biosoft, Cambridge, GBR). The dose-effect curves of each drug alone, and in combination, were produced by MTT assay. This data was entered into the CalcuSyn software and combination index (CI) values were graphed on fraction affected-CI (Fa-CI) plots. A $CI < 1$ is a synergistic interaction, $CI = 1$ is additive, and $CI > 1$ is antagonistic.

2.9. Statistical analysis

The MTT data are expressed as a mean ± SD. Statistical differences were determined by repeated measures one-way ANOVA where $p < 0.05$ was considered statistically significant.

Chapter 3: Results

3.1. A cytotoxic dose of cisplatin upregulates ATF3 protein expression

The *in vitro* model system used in this study comprised of five human cell lines, namely, WI38 (normal lung fibroblast), SKOV3 (ovarian adenocarcinoma), MCF7 (breast adenocarcinoma), PC3 (prostate adenocarcinoma), and A549 (NSCLC). In all cell lines examined, basal ATF3 protein levels are either very low or non-detectable. Upon treatment with a cytotoxic dose (8 μg/ml) of cisplatin for 24 hr, ATF3 protein expression is upregulated (Figure 3.1A). Verification of the sensitivity of all cell lines to increasing concentrations of cisplatin (0-

10 μg/ml) for 48 hr was determined by MTT assay (Figure 3.1B). Approximately 80% of cells survived a 2 μg/ml dose of cisplatin, whereas only 25-55% survival was observed with the 8 μg/ml dose of cisplatin.

A time-course experiment was conducted to gain insight into the kinetics of ATF3 protein expression upon cisplatin treatment (Figure 3.2). Samples were collected at four time points (6, 18, 24, 48 hr) and ATF3 protein expression was evaluated by Western blot. No significant ATF3 induction was detected in any cell line treated with the non-cytotoxic dose of cisplatin (2 μg/ml). All five cell lines treated with the cytotoxic dose of cisplatin (8 μg/ml) demonstrated varying degrees of ATF3 induction. The normal fibroblast cell line showed its strongest induction at 48 hr, whereas the cancer cell lines peaked at 18 and 24 hr. The localization of ATF3 protein within the cell was determined by IHC (Figure 3.3). ATF3 protein was not detected in untreated MCF7 and PC3, but after 24 hr of 8 μg/ml cisplatin exposure, nuclear ATF3 protein expression is observed.

3.2. Downstream targets of ATF3 transcriptional regulation

As a transcription factor, ATF3's function is to modulate the expression of downstream targets by binding to their promoters and activating or inhibiting transcription. Since ATF3 has been shown to downregulate cell cycle regulators (87, 116-118) and upregulate apoptotic factors (106, 107, 163), we hypothesized that ATF3 is important for cisplatin's cytotoxic action in cancer cells. Cisplatin's cell cycle inhibitory and apoptotic effects may be mediated in part by ATF3's binding and regulation of the *cyclin D1* and *CHOP* gene promoters, respectively. The expression of potential ATF3 targets in response to cisplatin treatment was determined by

Figure 3.1. ATF3 expression and cisplatin sensitivity in a panel of cell lines. **A**, Four cancer cell lines (SKOV3, MCF7, A549, PC3) and a normal lung fibroblast cell line (WI38) were treated with 2 μg/ml and 8 μg/ml cisplatin (CDDP) for 24 hr. ATF3 protein was detected by Western blot and actin was used as a loading control. **B**, MTT assay assessing the cell lines' sensitivity to increasing concentrations (0-10 μg/ml) of cisplatin over a 48 hr period. Data are represented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars are representative of six technical replicates. The experiment was repeated with similar results.

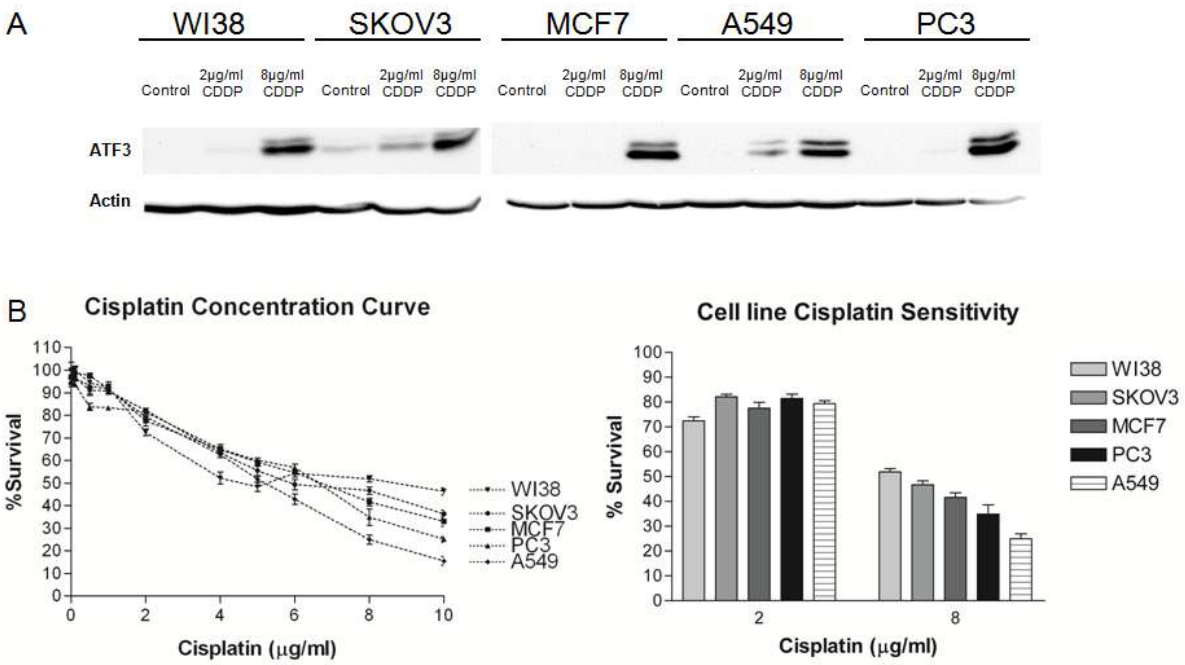

A
WI38
SKOV3
MCF7
A549
PC3
Control
2μg/ml CDDP
8μg/ml CDDP
ATF3
Actin
B
Cisplatin Concentration Curve
%Survival
Cisplatin (μg/ml)
WI38
SKOV3
MCF7
PC3
A549
Cell line Cisplatin Sensitivity
% Survival
Cisplatin (μg/ml)

Figure 3.2. Cisplatin-induced ATF3 protein expression over time. Western blot analysis of ATF3 protein expression in WI38, SKOV3, MCF7, PC3, A549 cells treated with cisplatin (2 and 8 μg/ml) at 6, 18, 24, 48 hr time points. The cytotoxic dose (8 μg/ml) of cisplatin induces ATF3 protein expression in all five cell lines. Actin is used as a loading control. The experiment was repeated with similar results.

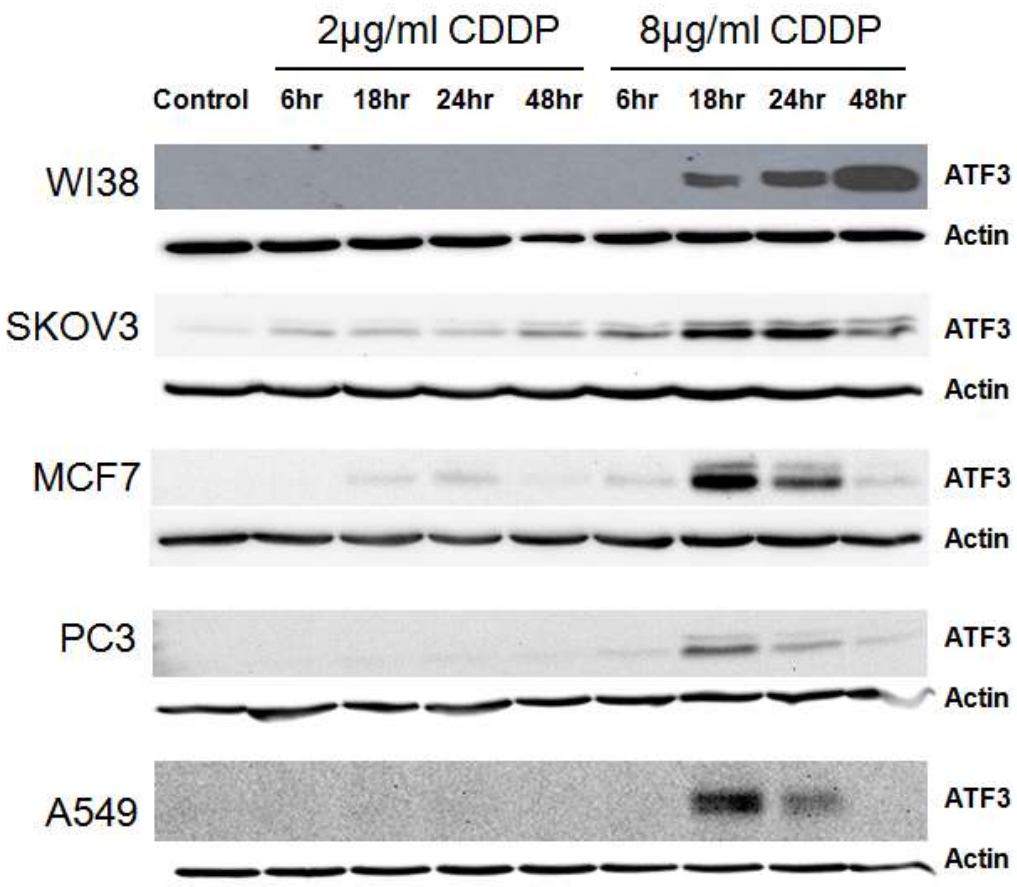
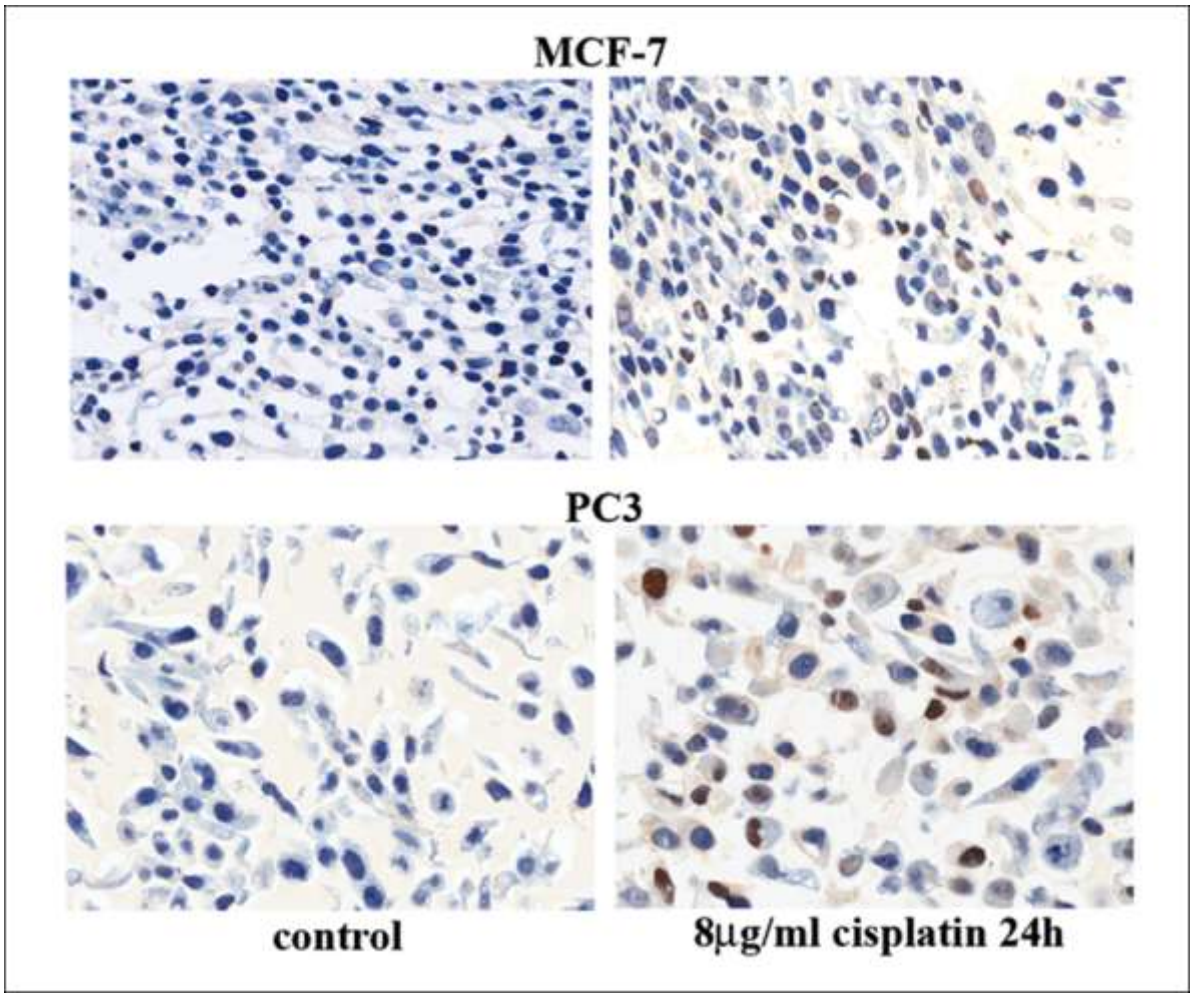

2μg/ml CDDP
8μg/ml CDDP
Control
6hr
18hr
24hr
48hr
6hr
18hr
24hr
48hr
WI38
ATF3
Actin
SKOV3
ATF3
Actin
MCF7
ATF3
Actin
PC3
ATF3
Actin
A549
ATF3
Actin

Figure 3.3. Immunohistochemical analysis of ATF3 expression. MCF7 and PC3 cell lines were either untreated (control) or treated with 8 µg/ml cisplatin for 24 hr. Cisplatin-treated cells show nuclear ATF3 staining (164). Hematoxylin blue stain is used as a nuclear counterstain.


MCF-7
PC3
control
8μg/ml cisplatin 24h

Western blot (Figure 3.4). Thapsigargin (1 μM, 24 hr) was used as a control since it is known to activate ATF3 and CHOP and downregulate cyclin D1 through the ISR pathway (107, 165).ATF3 protein expression was increased in a dose-dependent manner upon exposure to cisplatin (1, 2, 5, 8, 10, μg/ml) for 24 hr in all cell lines examined except WI38, which peaked at 5 μg/ml. Correspondingly, cyclin D1 protein expression, a cell cycle activator, was reduced at the same concentrations, suggesting a reduction in cell cycle progression. CHOP, a pro-apoptotic factor, showed increased protein expression in the SKOV3 and A549 cell lines.

A ChIP assay was performed in the cancer cell lines to determine if the modulation of protein expression was due to direct promoter binding. In all cell lines examined, ATF3 was bound to the *CHOP* gene promoter upon treatment with the highest dose of cisplatin (8 μg/ml) (Figure 3.5). In the cell lines that showed cisplatin-induced CHOP protein (SKOV3 and A549), more *CHOP* promoter DNA was pulled down with the ATF3 antibody upon 8 μg/ml cisplatin exposure than with 5 μg/ml cisplatin or with the untreated control. However, in the cell lines that did not produce a robust induction of CHOP protein (MCF-7 and PC3), a weak association of the ATF3 protein and *CHOP* gene promoter DNA was observed with 8 μg/ml cisplatin, suggesting that only undetectable levels of CHOP protein were induced.

3.3. Enhancing ATF3 expression may potentiate cisplatin cytotoxicity

The Dimitroulakos lab has shown that a lung cancer cell line (A549) with reduced ATF3 expression by shRNA is more resistant to the cytotoxic effects of cisplatin compared to controls (113). We have also shown that cisplatin in combination with an ATF3-inducing HDAC inhibitor is able to sensitize cells to cisplatin treatment (108). Our goal is to identify novel platin-based combinational therapeutics by enhancing the induction of ATF3 through

Figure 3.4. Protein expression of potential ATF3 targets. Western blot analysis of ATF3, cyclin D1 and CHOP protein in a panel of cell lines following 24 hr treatment with increasing concentrations (1 - 10 μg/ml) of cisplatin. Actin is used as a loading control and 1 μM thapsigargin (Tg), a known ER stressor, is used as a positive control. In the ovarian (SKOV3) and lung (A549) cell lines, cyclin D1 downregulation is correlated with ATF3 and CHOP upregulation. In the breast (MCF7), prostate (PC3) and normal lung (WI38) cell lines cyclin D1 downregulation is correlated with ATF3 upregulation but CHOP expression is not detected. The experiment was repeated with similar results.

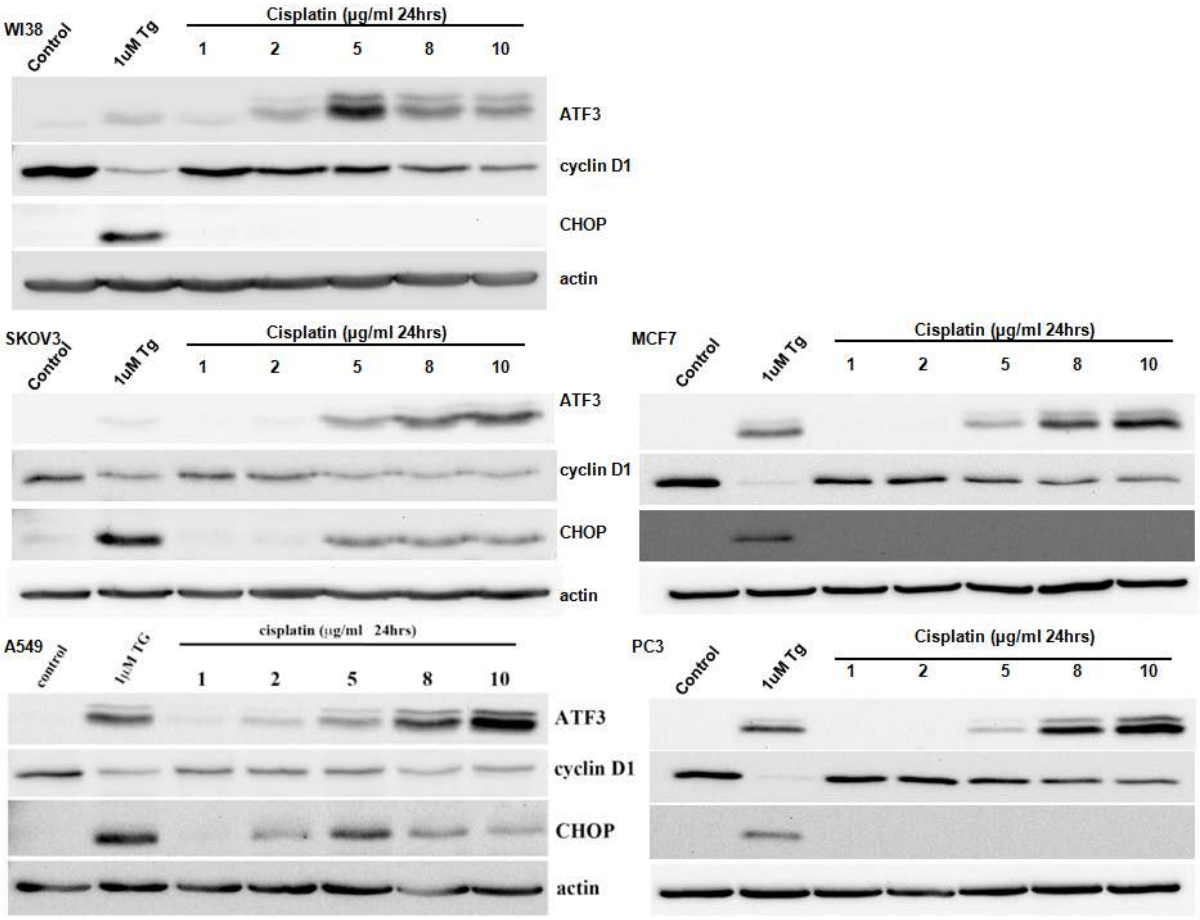
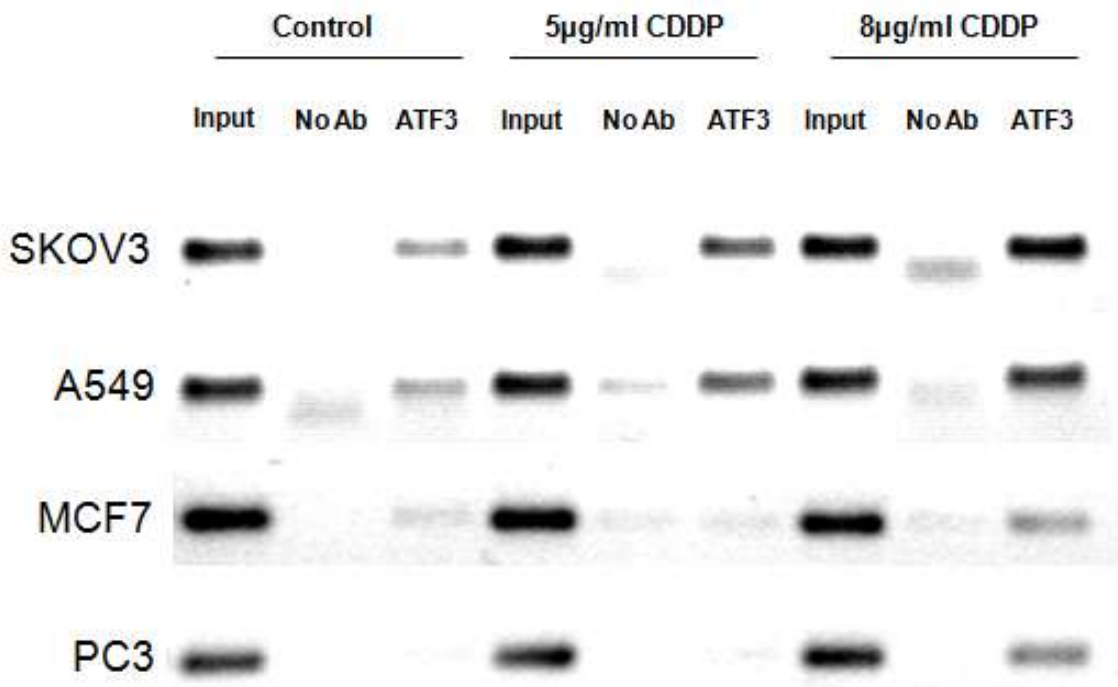

WI38
Control
1uM Tg
Cisplatin (μg/ml 24hrs)
1
2
5
8
10
ATF3
cyclin D1
CHOP
actin
SKOV3
Control
1uM Tg
Cisplatin (μg/ml 24hrs)
1
2
5
8
10
ATF3
cyclin D1
CHOP
actin
MCF7
Control
1uM Tg
Cisplatin (μg/ml 24hrs)
1
2
5
8
10
A549
control
1μM TG
cisplatin (μg/ml 24hrs)
1
2
5
8
10
ATF3
cyclin D1
CHOP
actin
PC3
Control
1uM Tg
Cisplatin (μg/ml 24hrs)
1
2
5
8
10

Figure 3.5. ATF3 binds the* CHOP *gene promoter. ChIP assay of cancer cell lines treated with 5 µg/ml or 8 µg/ml cisplatin (CDDP) for 24 hr. Formalin-fixed cell lysates were sonicated and incubated with an ATF3 antibody overnight. The antibody was pulled down using agarose beads, crosslinking was reversed and the DNA was purified. PCR amplification using primers specific to the *CHOP* gene promoter was performed and PCR products were run on a 1.6% agarose gel.


Control
5μg/ml CDDP
8μg/ml CDDP
Input
No Ab
ATF3
Input
No Ab
ATF3
Input
No Ab
ATF3
SKOV3
A549
MCF7
PC3

alternate mechanisms. Thus, a high throughput drug screen of potential enhancers of cisplatin cytotoxicity was performed. Two cancer cell lines, PC3 and A549, were treated with a chemical library of 1200 FDA approved compounds, alone or in combination with cisplatin (Figure 3.6). MTT assay results showed increased cisplatin cytotoxicity when combined with clinically approved chemotherapy agents such as topotecan, gemcitabine and docetaxel, which are currently used in the treatment of ovarian cancer and NSCLC (Figure 3.7A). The HDAC inhibitor vorinostat also showed a combined effect with cisplatin, validating our previous results in a lung cancer cell line model (Figure 3.7B) (108). Disulfiram, marketed as Antabuse and used for alcohol aversion therapy, increased cisplatin's cytotoxic action in both cell lines examined (Figure 3.8A). This novel finding was validated in four cell lines by MTT assay using varying concentrations of disulfiram (100nM, 150nM) and cisplatin (0-10 μg/ml) (Figure 3.8B).

The combination of disulfiram and cisplatin greatly increased the cytotoxicity of cisplatin alone, and this was determined to be statistically significant in all cell lines ($p < 0.001$). To understand the nature of the combination effect, the Chou-Talalay method was used to distinguish between additive and synergistic interactions (162). Three of the four cell lines were evaluated since the PC3 cell line was too sensitive to the combination treatment to produce reliable results (Figure 3.9). The potency and shape of the dose-effect curves of disulfiram and cisplatin alone, and in combination, were analyzed by CalcuSyn software. Fa-CI plots were generated to show the combination effect. CI values of 1 are additive, less than 1 are synergistic and more than 1 are antagonistic. In all cell lines examined, the combination of disulfiram and cisplatin were synergistic at high doses of cisplatin treatment.

Figure 3.6. Prestwick library screen. A given cancer cell line was exposed to 1200 FDA approved compounds at 1 μM concentration for 72 hr. One compound per well amounted to 15 x 96 well plates. The cell line was pre-treated with the drug library for 24 hr and then exposed to 4 μg/ml cisplatin for 48 hr. Cell viability was assessed by the MTT assay where the untreated control cells were taken to be 100%. The red circle shows a single agent that kills cancer cells.

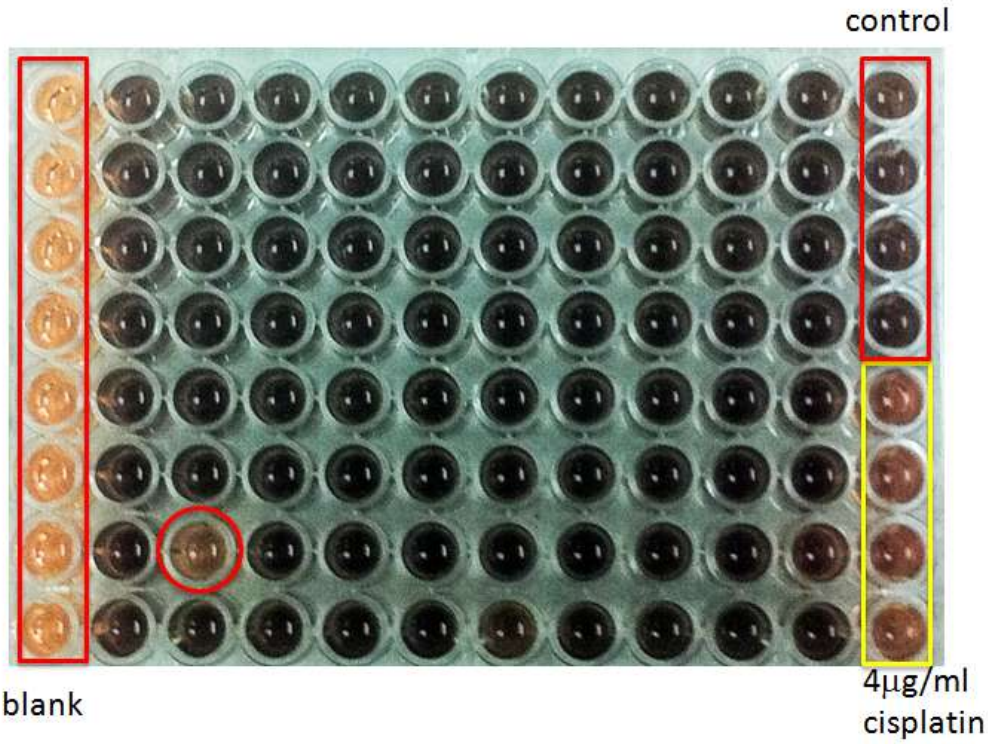

control
blank
4μg/ml
cisplatin

Figure 3.7. FDA approved compounds enhance cisplatin cytotoxicity. MTT assay showing that chemotherapy agents (166) and vorinostat (**B**) potentiate cisplatin cytotoxicity in PC3 and A549 cells. ■ represents a drug in the library screen exposed to cells for 72 hr at 1μM concentration. The dotted line shows the percent viability of the cell line treated with 4 μg/ml cisplatin for 48 hr. ♦ represents a drug in the library screen that was pre-treated at 1μM for 24 hr, followed by 48 hr treatment of 4 μg/ml cisplatin.

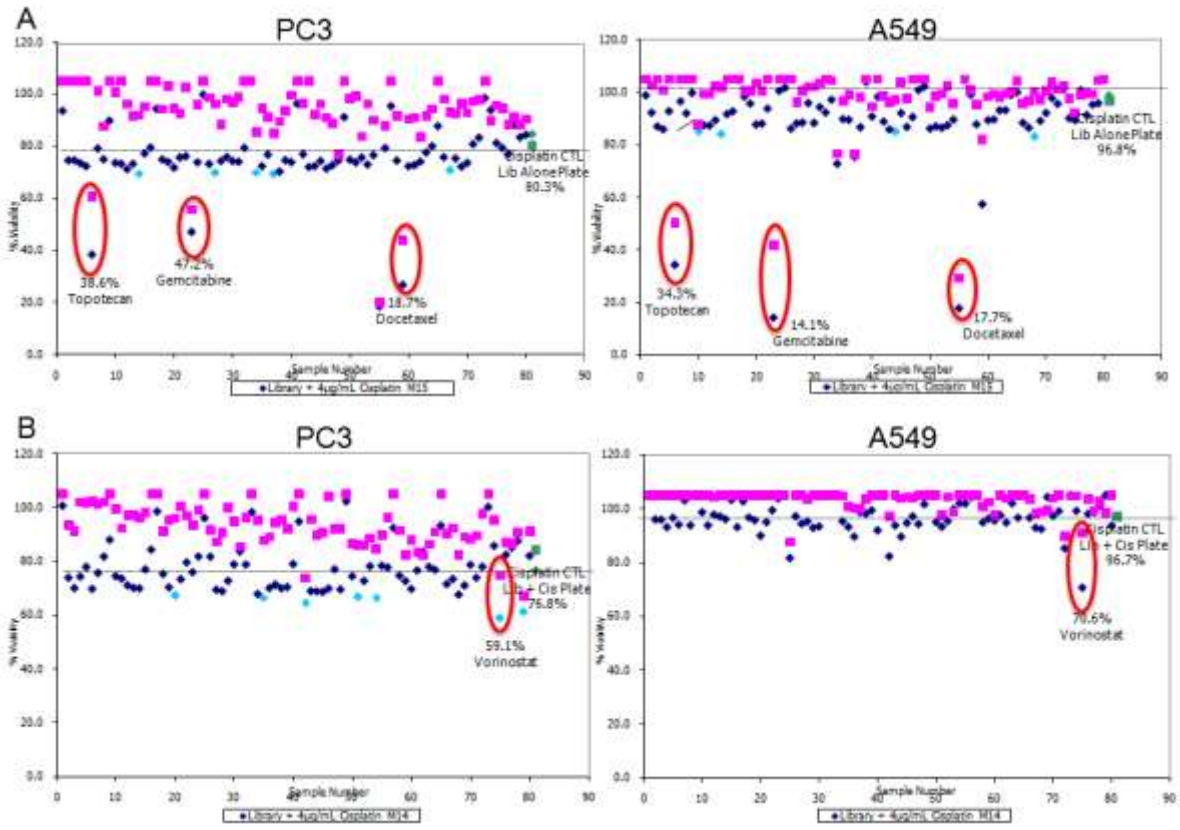
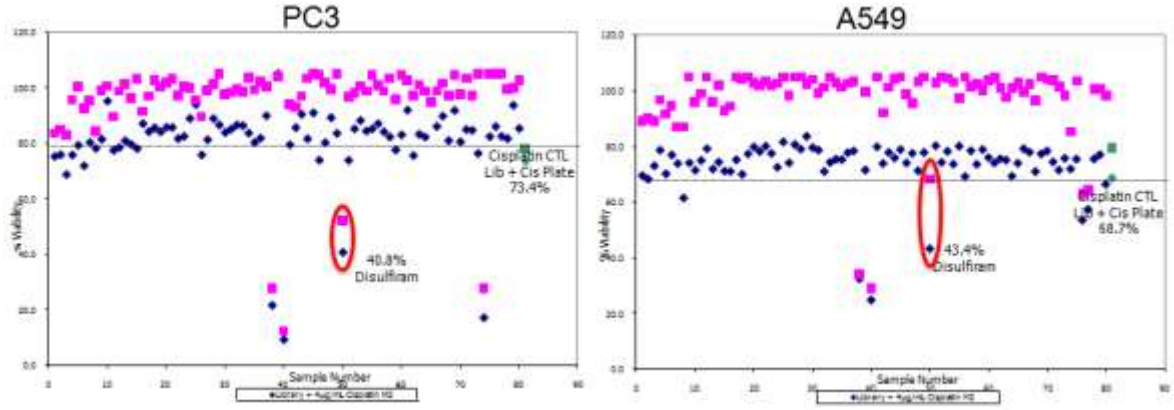

A
PC3
A549
38.6%
Topotecan
47.2%
Gemcitabine
18.7%
Docetaxel
Cisplatin CTL
Lib Alone Plate
80.3%
34.3%
Topotecan
14.1%
Gemcitabine
17.7%
Docetaxel
Cisplatin CTL
Lib Alone Plate
96.8%
% Viability
Sample Number
Library + 4µg/mL Cisplatin M15
B
PC3
A549
59.1%
Vorinostat
Cisplatin CTL
Lib + Cis Plate
75.8%
70.6%
Vorinostat
Cisplatin CTL
Lib + Cis Plate
96.7%
% Viability
Sample Number
Library + 4µg/mL Cisplatin M14

Figure 3.8. Disulfiram enhances the cytotoxicity of cisplatin. **A**, MTT assay showing PC3 and A549 cells pre-treated with 1 μM drug library for 24 hr with (♦) or without (■) 48 hr treatment of 4 μg/ml cisplatin. **B**, MTT assay assessing the cell lines' sensitivity to 24 hr pre-treatment of 0 nM (black), 100 nM (dark grey) or 150 nM (light grey) disulfiram followed by increasing concentrations (0-10 μg/ml) of cisplatin over a 48 hr period. Differences in % viability between cisplatin alone (black) and 100 nM (dark grey) or 150 nM (light grey) disulfiram combination treatment are statistically different in all cell lines (one-tailed *t* test, $p < 0.001$). Data are represented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars are representative of six technical replicates. The experiment was repeated with similar results.

A


PC3
A549
Cisplatin CTL
Lib + Cis Plate
73.4%
40.8%
Disulfiram
Cisplatin CTL
Lib + Cis Plate
68.7%
43.4%
Disulfiram
Sample Number

B

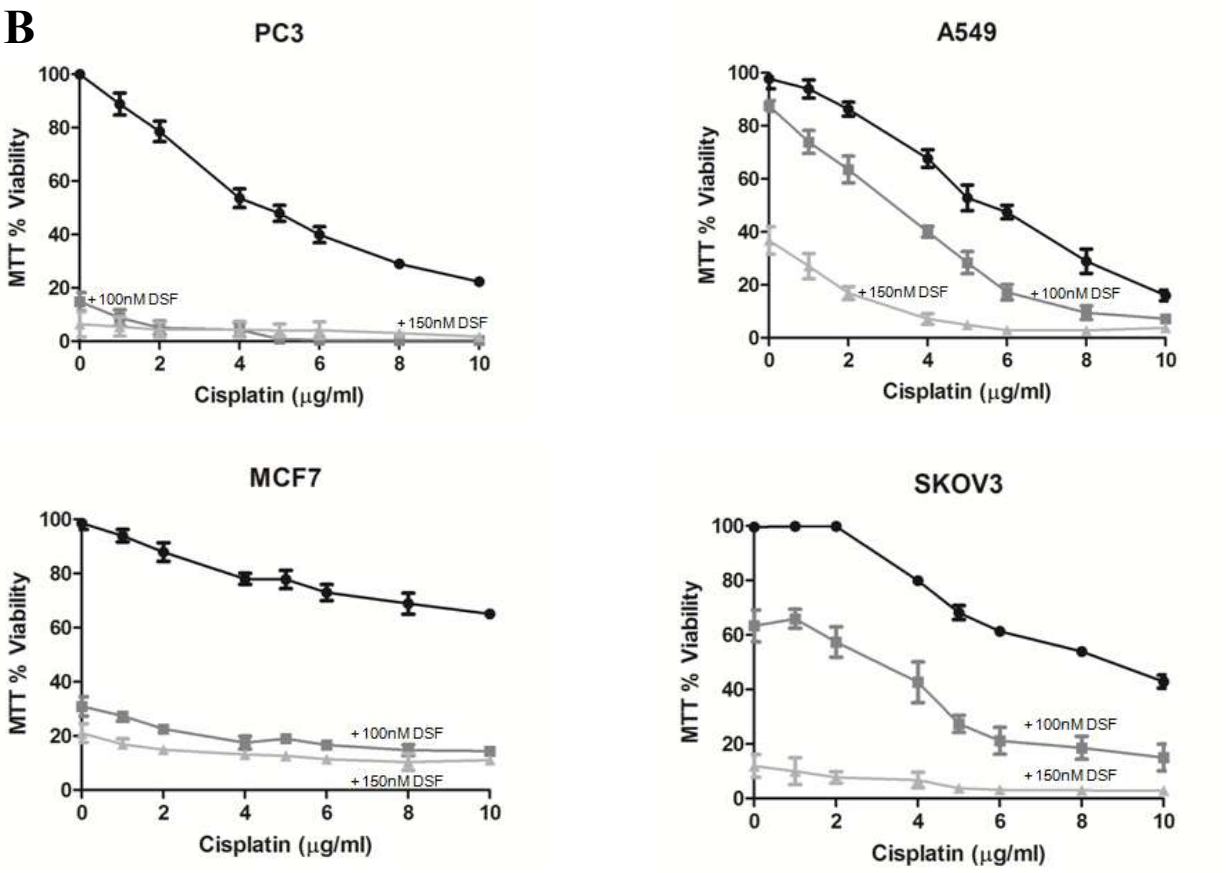
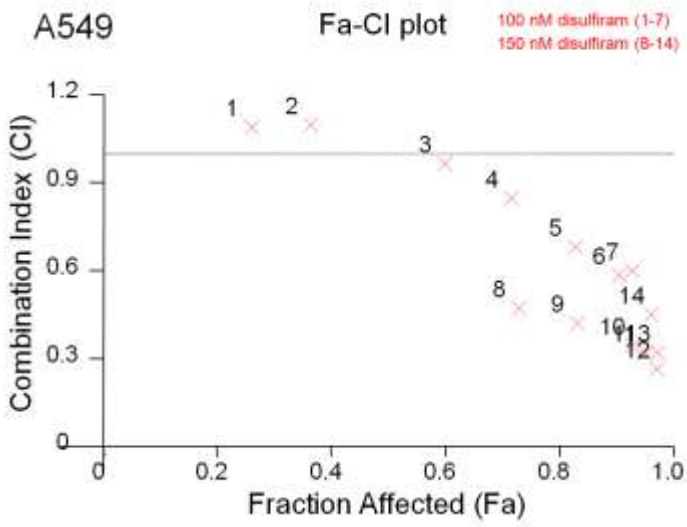
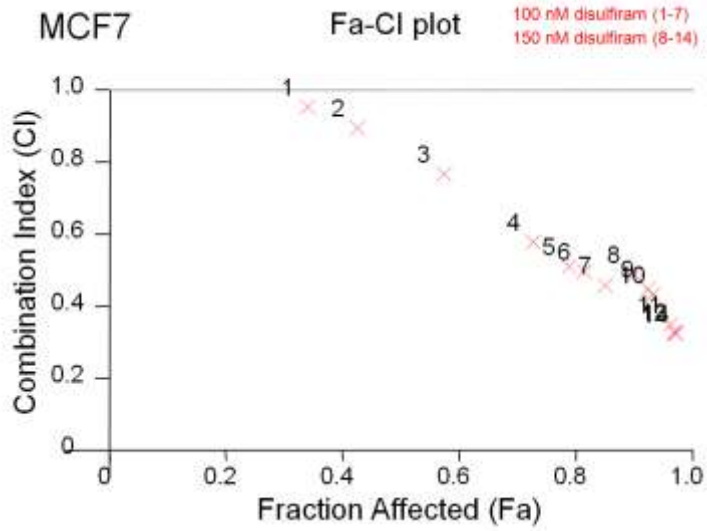
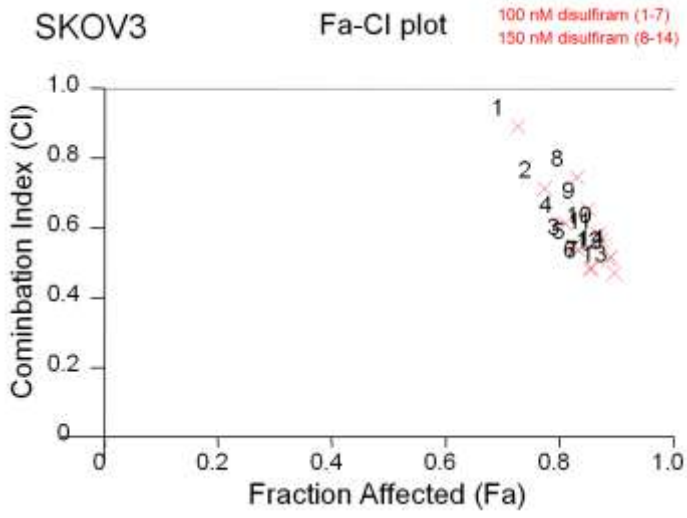

PC3
A549
MCF7
SKOV3
MTT % Viability
Cisplatin (μg/ml)
+100nM DSF
+150nM DSF

Figure 3.9. Disulfiram synergizes with cisplatin. Combination effect of cisplatin and disulfiram was evaluated by the Chou-Talalay method using CalcuSyn software. Fa-CI plots show the combination index values and the fraction affected at different concentrations of cisplatin (1, 2, 4, 5, 6, 8, 10 μg/ml) with 100 nM disulfiram (points 1-7) and 150 nM disulfiram (points 8-14). The additive effect of cisplatin and disulfiram is represented as CI = 1; CI < 1 indicates synergism; CI > 1 indicates an antagonistic interaction. Cisplatin and disulfiram combination therapy results in synergy in all cell lines over the higher doses of cisplatin evaluated.


A549
Fa-CI plot
100 nM disulfiram (1-7)
150 nM disulfiram (8-14)
Combination Index (CI)
Fraction Affected (Fa)
0
0.3
0.6
0.9
1.2
0.2
0.4
0.8
1.0


MCF7
Fa-CI plot
100 nM disulfiram (1-7)
150 nM disulfiram (8-14)
Combination Index (CI)
Fraction Affected (Fa)
0
0.2
0.4
0.6
0.8
1.0


SKOV3
Fa-CI plot
100 nM disulfiram (1-7)
150 nM disulfiram (8-14)
Cominbation Index (CI)
Fraction Affected (Fa)
0
0.2
0.4
0.6
0.8
1.0

Loss of cell viability observed in the MTT assay was likely due to apoptosis since PARP cleavage was detected by Western blot in two cell lines (PC3 and SKOV3) (Figure 3.10). The PC3 cell line exhibited PARP cleavage after 48 hr treatment with disulfiram (100 nM, 150 nM) and 24 hr treatment with cisplatin (8 μg/ml). When pre-treated with disulfiram and then combined with cisplatin, PARP cleavage was enhanced. In the more cisplatin- and disulfiram-resistant SKOV3 cell line, PARP cleavage was observed with increasing concentrations of disulfiram treatment alone, and the combination treatment also showed PARP cleavage. This provides evidence that the combination of disulfiram and cisplatin causes apoptosis in human cancer cell lines.

It was also observed that disulfiram treatment upregulated ATF3 protein expression (Figure 3.10). In the PC3 cell line, ATF3 protein expression was enhanced with the disulfiram and cisplatin combination treatment. Disulfiram alone upregulated ATF3 protein expression in the SKOV3 cell line and ATF3 was also enhanced with the disulfiram and cisplatin combination treatment.

3.4. ATF3 mRNA and protein expression in patient tumour samples

Since ATF3 plays a role in the cellular response to cisplatin chemotherapy, it may act as a potential indicator to predict a patient's sensitivity to cisplatin. Ovarian and lung TMAs were stained to assess ATF3 protein expression in a large number of tumour samples from diverse pathological origins. Overall, ATF3 protein was highly expressed in the ovarian (69%) and lung (80%) tumour tissues (Figure 3.11). Of the positive samples, only 19% of ovarian and 18% of lung tumours expressed ATF3 in the nucleus.

When the tumours were divided into histological subtype, ATF3 nuclear expression was most prominent in the mucinous samples compared with the endometrioid and serous ovarian

Figure 3.10. Disulfiram induces ATF3 protein and PARP cleavage. Western blot analysis of PARP cleavage and ATF3 protein upon treatment with 100 nM and 150 nM disulfiram (48 hr), 8 μg/ml cisplatin (48 hr), and pre-treatment with disulfiram (24 hr) followed by concurrent disulfiram and cisplatin treatment (24 hr). Actin is used as a loading control.

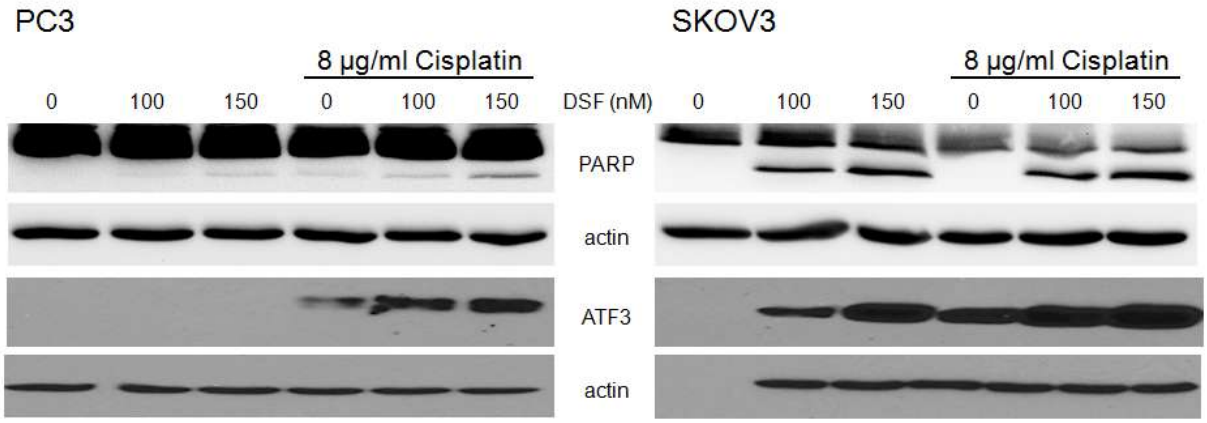
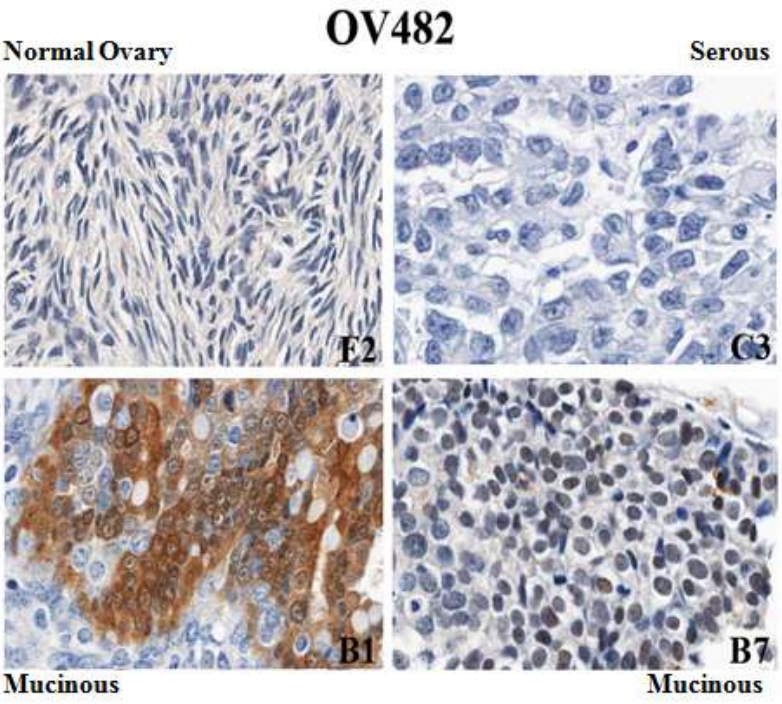
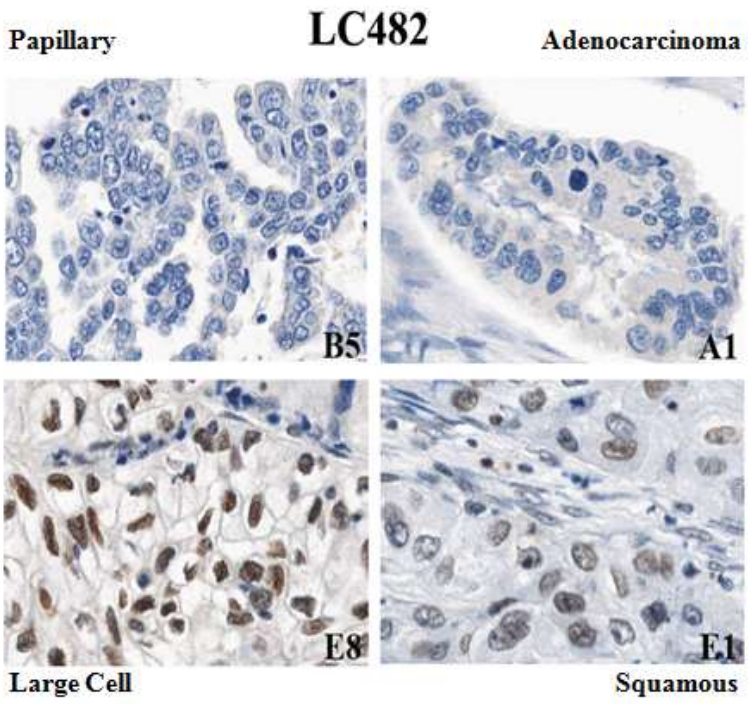

PC3
SKOV3
8 µg/ml Cisplatin
8 µg/ml Cisplatin
0
100
150
0
100
150
DSF (nM)
0
100
150
0
100
150
PARP
actin
ATF3
actin

Figure 3.11. ATF3 protein is differentially expressed in ovarian and lung cancer tumours. Immunohistochemical analysis of ATF3 expression (164) in commercially available ovarian (OV482) and lung (LC482) cancer tissue microarrays, counterstained in hematoxylin.


OV482
Normal Ovary
Serous
E2
C3
B1
B7
Mucinous
Mucinous


LC482
Papillary
Adenocarcinoma
B5
A1
E8
E1
Large Cell
Squamous

subtypes (Table 2). The lung adenocarcinoma and squamous cell carcinoma subtypes expressed relatively similar percentages of nuclear ATF3. To validate these results and expand the study, patient samples with available clinical data would need to be correlated with ATF3 expression. This would determine if ATF3 could be used in the clinic to identify subgroups of patients who would benefit from cisplatin treatment versus other treatment options.

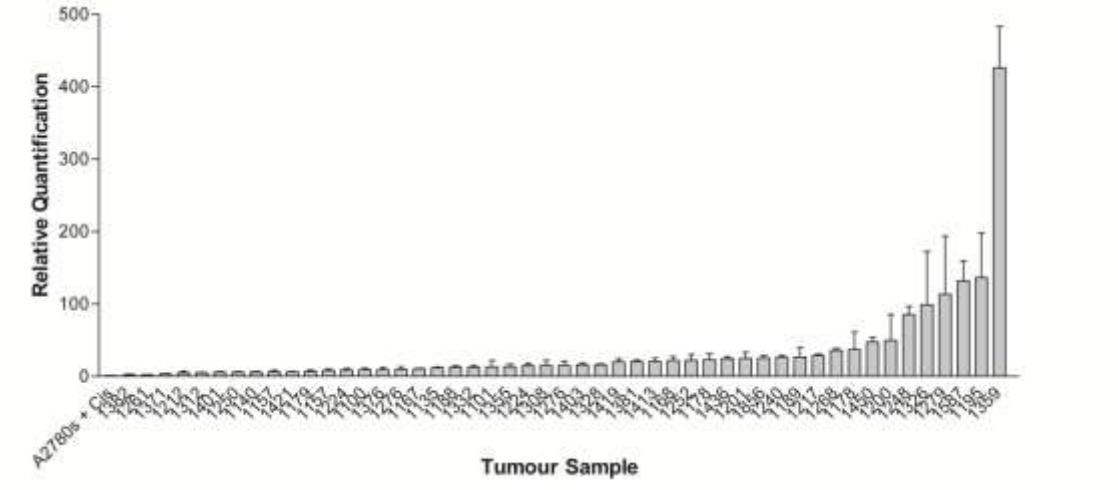
Forty nine serous ovarian tumours from the Ovarian Cancer Tissue Bank at the OHRI were available to compare ATF3 mRNA levels with protein expression detected by IHC. Total RNA was extracted and ATF3 mRNA expression was analyzed by quantitative RT-PCR. Variable ATF3 mRNA expression was observed, with a small subset of tumours showing a strong expression of ATF3 compared to an ovarian cancer cell line (A2780s) treated with 2 μg/ml cisplatin for 24 hr (Figure 3.12). The high mRNA expression reflects what is seen with the positive nuclear protein staining observed in the serous ovarian cancer subtype (Table 2).

Table 2. ATF3 protein staining of ovarian and lung cancer TMAs

Ovarian Cancer Tissue Microarrays		**Lung Cancer Tissue Microarrays**	
Pathology	ATF3 positive cores (cytoplasm and/or nucleus)	Pathology	ATF3 positive cores (cytoplasm and/or nucleus)
Endometrioid	35/48 (73%)	Adenocarcinoma	49/69 (71%)
Mucinous	32/42 (76%)	Squamous cell carcinoma	67/75 (89%)
Serous	40/64 (62%)	**Total**	**116/144 (80%)**
Total	**107/154 (69%)**		

Ovarian Cancer Tissue Microarrays		**Lung Cancer Tissue Microarrays**	
Pathology	ATF3 positive cores (nucleus)	Pathology	ATF3 positive cores (nucleus)
Endometrioid	6/48 (12%)	Adenocarcinoma	10/69 (14%)
Mucinous	15/42 (36%)	Squamous cell carcinoma	16/75 (21%)
Serous	9/64 (14%)	**Total**	**26/144 (18%)**
Total	**30/154 (19%)**		

Figure 3.12. ATF3 mRNA expression levels in human ovarian tumour samples. Expression levels of ATF3 mRNA were determined in 49 serous epithelial ovarian carcinoma tumours by RT-PCR. The relative quantification of each tumour was determined by averaging three independent RNA extractions. The ovarian cell line A2780s, treated with 2 μg/ml cisplatin for 24 hr, was used to standardize the results and was given a value of 1. Fold differences in expression were calculated following normalization to GAPDH levels (ΔΔCt).


Relative Quantification
500
400
300
200
100
0
A2780s + Cis
Tumour Sample

Chapter 4: Discussion

Cisplatin is a potent cytotoxic agent that induces cell death by directly damaging DNA. However, the mechanism of tumour cell toxicity is not fully understood. Various cellular pathways, such as the MAPK cascades, have been implicated in regulating cisplatin's tumour cell killing. With the goal of improving patient response to cisplatin and overcoming tumour resistance, the investigation of stress pathways may reveal novel therapeutic avenues. In a previous study, ATF3 upregulation through the MAPK pathway was found to be an important regulator of cisplatin cytotoxicity (113). In our present study, ATF3 was shown to have a pro-apoptotic role in cisplatin response by directly binding to and activating the *CHOP* gene promoter, a key regulator of apoptosis. Cisplatin and disulfiram worked synergistically in combination to promote tumour cell toxicity *in vitro*, and ATF3 protein expression was induced by this combination therapy. Furthermore, in human tissue samples, ATF3 basal protein expression was highly expressed in a subset of ovarian and lung cancers. Overall, ATF3 plays an important role in the apoptotic response of cytotoxic agents and it may serve as a potential indicator of treatment outcome in a clinical setting.

4.1. Downstream targets of cisplatin-induced ATF3 activation

A cytotoxic dose of cisplatin induced ATF3 protein expression in a panel of cell lines. In the cancer cell lines, the induction of ATF3 was first observed at 6 hr, peaked from 18 to 24 hr and subsided by 48 hr. This observation is consistent with previous reports showing ATF3 upregulation at 24 hr by cisplatin, lovastatin and thapsigargin (88, 107, 113) and 14 hr by MG132 (94). However, the WI38 normal lung cell line showed the highest ATF3 protein

expression at 48 hr. This may be explained by the fact that WI38 is slightly more resistant to cisplatin than the other cell lines and is not undergoing as much cell death at the 48 hr time point.

ATF3 protein expression was localized in the nucleus of MCF7 and PC3 cells, consistent with its role as a transcription factor. The expression of ATF3 in these cells was heterogeneous, which might be a result of oscillation with the cell cycle. ATF3 was found to be upregulated during S phase (98, 99) and its gene promoter has both Myc/Max and E2F transcription factor binding sites, which are implicated in cell cycle regulation (71).

We determined that with increasing concentrations of cisplatin, ATF3 induction correlated with a reduction in cyclin D1 protein expression. This supports work by Lu *et al.,* which showed that ATF3 directly represses cyclin D1, leading to cell cycle arrest at the G1-S checkpoint (87). ATF3 induction also correlated with an upregulation of the pro-apoptotic protein CHOP in the SKOV3 and A549 cell lines and ATF3 was shown to directly bind the *CHOP* gene promoter in all four cell lines examined. This is the first report to show that outside of the ISR pathway, which is not involved in ATF3 induction by cisplatin (113), ATF3 can directly activate the transcription of *CHOP*. All other studies showed an inhibitory effect upon physiological stress (82, 86) and arsenite-induced stress (119). Therefore, ATF3 has the ability to both activate and repress the same promoter. The distinction might be due to which cofactor ATF3 is paired with and which promoter elements are bound. This is the case for the *KAI1* gene promoter where ATF3 activates its transcription when bound to JunB, but represses its transcription when bound to NF-κB (167).

ATF3 has also been shown to induce apoptosis through the activation of other pro-apoptotic factors. Noxa, a Bcl-2 homology3 (BH3)-only pro-apoptotic protein, is a direct target of ATF3 and is important for ATF3-mediated apoptosis by the new anti-cancer agent human

Galectin-9 (hGal9) (163), by ERAD inhibitors (168), and upon inflammation in islet cells (169). ATF3 has also been linked to the activation of the extrinsic apoptotic pathway through the direct transcriptional activation of the hypoxia inducible factor (*Hif)-2α* gene followed by downstream activation of TNF- related apoptosis-inducing ligand (TRAIL) and caspase 7 (111).

Although our group has observed ATF3 as a pro-apoptotic factor involved in cisplatin-induced cytotoxicity, many other groups have shown a pro-survival role for this transcription factor. This apparent discrepancy can partially be explained by ATF3's involvement with other proteins that have dichotomous roles in cell proliferation. ATF3 is directly activated by c-Myc, which is well known as an oncogene that promotes cell growth and survival (170). However, c-Myc can also have pro-apoptotic activity and can act as a tumour suppressor (171). The same phenomenon is true for TGFβ; it induces cell cycle arrest and apoptosis in non-malignant cells but increases cell motility and metastasis in malignant cells (132). ATF3 induction by TGFβ has lead to cell cycle arrest through the inhibition of Id-1 and to enhanced cell motility through the upregulation of EMT genes (117, 140). As a positive regulator of p53, ATF3 can facilitate the various roles of p53 in the cell such as DNA repair, cell cycle inhibition, and apoptosis (89, 172). Therefore, ATF3's ability to interact with numerous proteins involved in different cellular mechanisms can explain the pleiotropic effects that ATF3 can have on cellular functions.

4.2. Potentiation of cisplatin cytotoxicity

Our group screened a chemical library for compounds that synergized with cisplatin, and disulfiram emerged as a promising candidate. Disulfiram is a member of the dithiocarbamate class of chemicals, which complex with transition metals and are used as vulcanizing and analytical agents in the field of chemistry (145). However, their use has expanded dramatically

in the last few decades (145). Disulfiram was first used as a pesticide in the 1930s because it chelates copper, which is necessary for the respiratory chain of primitive animals (173). In the 1940s, it was marketed as Antabuse for the treatment of chronic alcoholism by preventing the complete metabolism of alcohol through the inhibition of the human liver aldehyde dehydrogenase (ALDH) enzyme (145, 174). Disulfiram can also inhibit the metabolism of dopamine and is currently in development for the management of cocaine abuse (175). Long term studies show that continued use of disulfiram is safe, with liver damage being the most common side effect (176).

Disulfiram was first shown to have an anti-cancer effect in the 1970s although its mechanism of action was unknown (166). In the last decade, a number of studies have begun elucidating disulfiram's role in suppressing cancer cell growth. Many cellular targets involved in multidrug resistance, angiogenesis and invasion have been described (177). However, its ability to inhibit the proteasome has risen to the forefront of research in the last few years. In 2006, two studies demonstrated disulfiram's proteasome inhibitory effects coupled with suppression of tumour growth in a breast and leukemia model (178, 179). This effect has been expanded to include efficacy in a wide range of cancer cells and patient-derived tumour cells with disulfiram being most active in haematological, ovarian, breast, prostate and NSCLC cancers (180-182). Importantly, disulfiram appears to be selective for cancer cells due to their higher intracellular copper concentrations (178). This selectivity was shown in prostate cancer versus normal prostate epithelial cells (182), melanoma versus melanocytes (183), leukemia versus peripheral blood mononuclear cells (179), and breast cancer versus normal breast cells (178). Several clinical trials are evaluating disulfiram as a single-agent treatment in metastatic melanoma [NCT00256230] and prostate cancer [NCT01118741], and as combination therapy with cisplatin

in lung cancer [NCT00312819]. Thus, disulfiram's ability to inhibit the proteasome along with its safety profile makes it an exciting candidate as an anti-cancer agent.

The dose-effect curves of disulfiram and cisplatin treatment were generated by the MTT assay, a colorimetric assay used to measure cell viability (184). It was shown in the 1980s that the *in vitro* determination of drug sensitivity by the MTT assay correlated well with a patient's response in clinical practice (185). It was concluded that the MTT assay was a good approach for screening human tumour cell lines to new drugs. This is because the dose-response curves highly correlate with both the clonogenic and dye-exclusion assays in a number of models (184). Specifically, the assessment of cisplatin cytotoxicity by MTT assay correlates with the clonogenic assay in ovarian cancer and HNSCC cells (186, 187). However, it is acknowledged that the colony forming assay is the most sensitive method of detecting cell viability since it excludes viable but non-proliferating or slowly proliferating cells (187). The MTT assay is based on the mitochondrial metabolic activity of cells (184). The limitation of using the MTT assay as a measure of cell viability is that it measures non-dividing, cytostatic cells that have functional metabolic activity (187). In addition, reduction of the MTT reagent can be perturbed by drugs that have an effect on the mitochondria or on redox reactions within the cytoplasm. Therefore, it is important to measure cell viability by more than one biological assay, which is why we assessed the cleavage of PARP by Western blot analysis. PARP is a DNA damage repair protein that is a direct target of caspases upon the initiation of the apoptotic cascade (51). Therefore, detecting cleavage products of PARP is indicative of cells undergoing apoptosis.

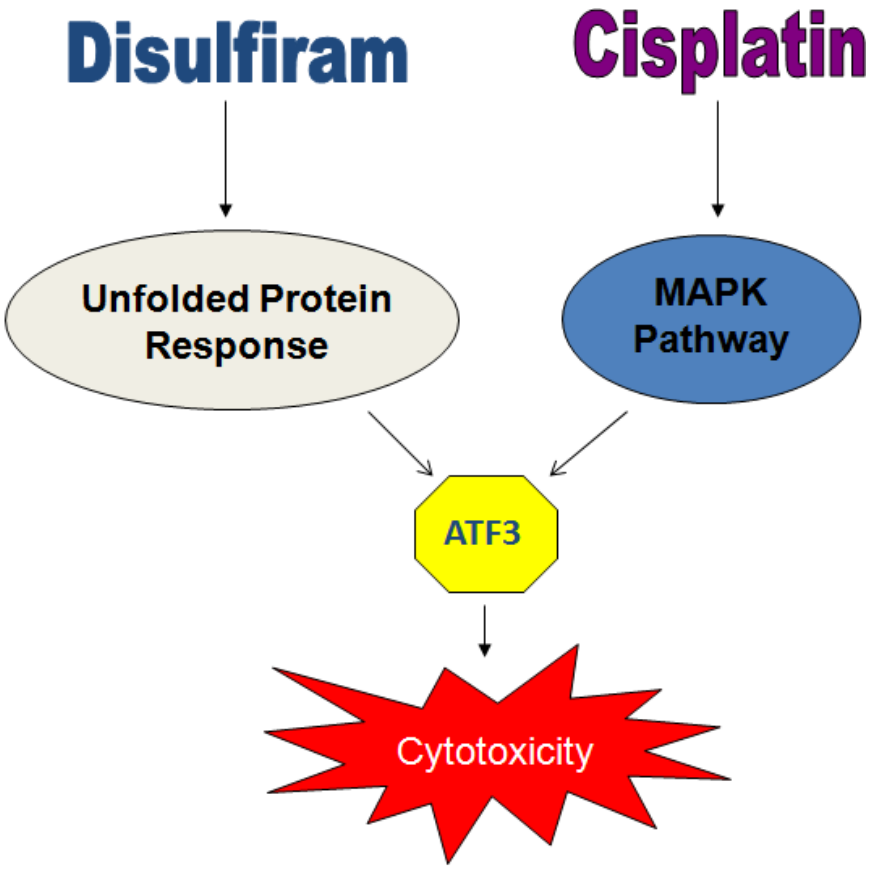
Since proteasome inhibitors are known to induce the ISR pathway (151-153), an upstream induction pathway of ATF3, the involvement of ATF3 in disulfiram-induced apoptosis was investigated. ATF3 protein expression was greatly upregulated upon combination treatment

compared to disulfiram or cisplatin treatment alone (Figure 3.10). This suggests that ATF3 is being induced by two separate pathways, creating a synergistic effect on its protein expression (Figure 4.1). The concept of synergism through ATF3 has been previously shown. An HDAC inhibitor that upregulates ATF3 through the ISR pathway potentiated cisplatin cytotoxicity in a lung cancer cell model (108). ATF3 was important for cisplatin-induced cell killing since knockdown of ATF3 by shRNA attenuated cisplatin's toxicity. As well, overexpression of ATF3 by a tetracycline-inducible system enhanced the growth suppression of cisplatin, IR and etoposide (103). Curcumin, an anti-cancer agent, also upregulates ATF3 in a pro-apoptotic manner and overexpression of ATF3 synergized with curcumin *in vitro* (135, 188). In addition, disulfiram has been shown to synergize with gemcitabine, which is also an activator of ATF3 (189, 190). Therefore, there are adequate background studies to support the hypothesis that ATF3 is partially responsible for the synergism observed between disulfiram and cisplatin in this study.

4.3. ATF3 expression in patient tumour samples

Yin *et al.,* the only group to assess *ATF3*'s gene copy number in human cancer tissue, reported an 80% *ATF3* gene amplification rate in breast cancer tumour samples (128). However, another group reported a statistically significant reduction of ATF3 mRNA expression in all cancer tissues (17 types) compared to normal tissues (129). Specifically, ATF3 was downregulated in 80% of lung cancer, 60% of breast cancer and 50% of ovarian

Figure 4.1. Schematic model for enhancing cytotoxicity through ATF3. Disulfiram may upregulate ATF3 through the Unfolded Protein Response pathway and cisplatin upregulates ATF3 through the MAPK pathway. Combinational treatment of disulfiram and cisplatin converge on ATF3, leading to enhanced cytotoxicity.


Disulfiram
Cisplatin
Unfolded Protein Response
MAPK Pathway
ATF3
Cytotoxicity

cancer samples. The Cancer Genome Anatomy Project (http://cgap.nci.nih.gov) also concluded that ATF3 cDNA is significantly lower in cancer tissue versus normal tissue ($p<0.01$). Our study shows that only a small percentage of serous ovarian tumours express high levels of ATF3 mRNA, although we do not have the corresponding normal ovarian tissue to compare with. This highlights how difficult it can be to describe *ATF3* gene expression in general terms; its role in tumour development appears to be dependent on cell context.

Wang *et al.* examined ATF3 IHC staining in a human breast TMA (191). They showed that ATF3 nuclear expression was present in 18% of breast cancer samples, while many of the tumours expressed cytoplasmic staining (191). Cytoplasmic staining was also observed when the Santa Cruz ATF3 antibody, the same used in our and Wang's study, was properly optimized in breast cancer tissues (78). This confirms our TMA IHC findings; only 19% of ovarian and 18% of lung tumours exhibited nuclear staining but there was a high incidence of cytoplasmic staining (69% and 80% respectively). It would be interesting to determine if cytoplasmic ATF3 is trapped as a non-functional transcription factor in the cytosol or if it also participates in unknown activities outside the nucleus. ATF4 was shown to be negatively regulated by the intermediate filament vimentin, which bound ATF4 to prevent its translocation to the nucleus (192). In addition, when analyzing ATF3 expression in tumour samples it is important to note that the tumour microenvironment is under stressful conditions (118). Since ATF3 is downstream of the ISR pathway and this pathway is activated upon hypoxia and nutrient deprivation, it may contribute to the ATF3 overexpression seen in tumour samples (105).

It is interesting that the highest percentage of ATF3 positive nuclei was associated with the mucinous subtype of ovarian cancer compared to the endometrioid and serous subtypes. Of the epithelial ovarian cancers, mucinous cancers are the rarest, accounting for only 2-4% of

patients (193). Since most patients are diagnosed at stage I, they have a better prognosis than women with serous ovarian cancer who often present at a later stage. However, late stage mucinous cancers have a much worse prognosis; four times higher risk of death than serous cancer (194). This is likely because of an inherent platinum resistance, which might be due to specific genetic alterations that are unique to the mucinous subtype (194). Mucinous ovarian carcinomas have a higher rate of *KRAS* mutations and a lower rate of *BRCA* and *p53* mutations compared to serous tumours (193). Since activating *KRAS* mutations induce many signalling pathways, including the MAPK cascades, ATF3 might be preferentially activated in these tumours, explaining the higher incidence of nuclear ATF3 staining observed in our study. Clearly, new therapeutic strategies that overcome platinum resistance are required for this small but unique group of patients.

After initial analysis of ATF3 mRNA and protein in human patient samples, it is apparent that ATF3 expression is variable among tumours, although conclusions regarding tumour development and treatment strategies cannot be made. The next step would be to acquire tissue samples from cancer patients before and after treatment. It would be interesting to determine if an upregulation of ATF3 protein expression upon platinum treatment associates with increased efficacy of the drug. In conclusion, ATF3 is a potentially exciting predictor of patients' response to platinum chemotherapy. In fact, ATF3 over-expression has been shown to associate with prostate cancer samples that are resistant to standard androgen ablation treatment, showing a potential predictive role for ATF3 in that model (195).

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activating transcription factor 3 in the human prostate and its regulation by androgen in prostate cancer. *The Journal of urology* 175:1517-1522.

Contributions of Collaborators

- Immunohistochemical staining of the TMAs was performed by Louise Pelletier at the University of Ottawa Pathology Department and the slides were scored by Dr. Manijeh Daneshmand.

- Stefanie Reid performed the initial library drug screen on PC3 and A549 cells.

- Combination effect of disulfiram and cisplatin co-treatment was evaluated by Dr. Nima Niknejad using CalcuSyn software.

Appendix I: Ethics approval

Research Ethics Board approval for working with human ovarian cancer tumours: REB # 2007375-01H "Targeting BRCA1-related DNA repair to enhance chemosensitivity in sporadic epithelial ovarian cancer".

Curriculum Vitae

ANNA O'BRIEN

Education

Master of Science **2009-2011**
Biochemistry with a specialization in Human and Molecular Genetics
University of Ottawa, Ontario

- Award winner: Ontario Graduate Scholarship in Science and Technology, Sept 2010-present, $12,000

Bachelor of Science: Biochemistry Major **2001-2005**
McGill University, Montreal, Quebec
Graduated with Distinction

Publications

Weberpals J, **O'Brien A**, Niknejad N, Garbuio K, Clark-Knowles K, Dimitroulakos J. The effect of the histone deacetylase inhibitor M344 on BRCA1 expression in breast and ovarian cancer cells. *Cancer Cell International*. 2011, 11:29.

Weberpals J, Tu D, Squire J, Amin S, Islam S, Pelletier L, **O'Brien A**, Hoskins P, Eisenhauer E. BRCA1 expression as a prognostic marker in sporadic epithelial ovarian cancer: an NCIC CTG OV.16 correlative study. *Annals of Oncology*. 2011 doi: 10.1093/annonc/mdq770

Clark-Knowles K, **O'Brien A,** Weberpals J. BRCA1 as a therapeutic target in sporadic epithelial ovarian cancer. *Journal of Oncology*. 2010:891059.

St-Germain C, **O'Brien A**, Dimitroulakos J. Activating Transcription Factor 3 regulates in part the enhanced tumour cell cytoxicity of the histone deacetylase inhibitor M344 and cisplatin in combination. *Cancer Cell International*. 2010, 10:32

Weberpals J, Garbuio K, **O'Brien A**, Clark-Knowles K, Doucette S, Antoniouk O, Goss G, Dimitroulakos J. The DNA repair proteins *BRCA1* and *ERCC1* as predictive markers in sporadic ovarian cancer. *International Journal of Cancer*. 124: 806 – 815, 2009.

Conferences

O'Brien A, Dimitroulakos J. ATF3 as a novel regulator and predictor of cisplatin response in human cancer. American Association for Cancer Research – National Cancer Institute - European Organization for Research and Treatment of Cancer 22nd International Conference on Molecular Targets and Cancer Therapeutics. Berlin, Germany. Published Abstract and Poster Presentation. 2010

O'Brien A, Dimitroulakos J. ATF3 as a regulator and predictor of cisplatin response in human cancer. Biochemistry Microbiology and Immunology Department Poster Day. Ottawa, ON. Poster Presentation. 2010

Weberpals J, **O'Brien A**, Garbuio K, Clark-Knowles K, Dimitroulakos J. The effect of histone deacetylase inhibition on BRCA1 expression: A potential novel mechanism for enhancing platinum sensitivity in breast and

ovarian cancer. 9th Annual OHRI Research Day. Ottawa, ON. Award Recipient: 3rd place Master's Student Poster Presentation. 2009

Weberpals J, **O'Brien A**, Garbuio K, Clark-Knowles K, Dimitroulakos J. Histone Deacetylase Inhibition as a Potential Target of BRCA1 Expression: A Novel Mechanism to Enhance Platinum Sensitivity in Breast and Ovarian Cancer. 30th Annual General Meeting of the Society of Gynaecologic Oncologists of Canada. Vancouver, BC. Poster presentation. 2009

Weberpals J, Garbuio K, **O'Brien A**, Clark-Knowles K, Doucette S, Antoniouk O, Goss G, Dimitroulakos J. The evaluationof BRCA1 and ERCC1expression as predictors of platinum response in ovarian cancer. 29th Annual General Meeting of the Society of Gynaecologic Oncologists of Canada. Calgary, Ab. Poster Presentation. 2008

Garbuio K, **O'Brien A**, Weberpals J, Dimitroulakos J. Identification of ATF3 as a potential novel therapeutic target of ovarian carcinomas. 4th Canadian Conference on Ovarian Cancer Research. Montreal, Qc. Poster Presentation. 2008

Employment Experience

Senior Research Technician **2007-2011**
Ottawa Health Research Institute, Cancer Therapeutics, Ottawa, Ontario

- Coordinated and conducted extensive research in the treatment of Sporadic Epithelial Ovarian Cancer (SEOC) by examining the clinical relevance of gene and protein expression in patient tumour samples
- Developed a novel treatment strategy for SEOC by executing various laboratory experiments and participated in the writing and editing of research manuscripts

Research Technician **2006-2007**
StemPath Inc., Ottawa, Ontario

- Collaborated with a team of technicians and scientists to develop a novel therapeutic drug for cardiac and muscle regeneration
- Executed animal experiments and collected data for advancement into preclinical trials

Research Assistant **2000-2002**
Vascular Biology Laboratory, University of Ottawa Heart Institute, Ottawa, Ontario

- Researched angiogenesis in the scientific literature and contributed to discussions at group laboratory meetings
- Independently performed cell transformation assays as well as microscopy data analysis using Image-Pro software

Customer Service Representative **2005-2006**
Fisher Scientific, Ottawa, Ontario

- Offered customers proficient service by qualifying and supplying them with laboratory research and health care equipment
- Exhibited organizational skills by prioritizing work to meet deadlines and maintained excellent interpersonal skills with customers and co-workers

Pharmaceutical Sales Representative **2005**

IMPRESPharma, Gatineau, Quebec

- Independently and effectively promoted an asthma medication to French speaking physicians in the Gatineau area
- Displayed the ability to quickly learn the medication's mechanism of action, the disease it is treating, and the proper etiquette of sales

Volunteer Experience

Science Outreach Program Advisor **2009-2011**

Let's Talk Science Partnership Program, Ottawa, Ontario

- Partnered with elementary school teachers to encourage science literacy in their classrooms by leading hands-on activities.
- Lead groups of high school students through the Cancer Therapeutics Department and demonstrated key experiments important in researching the cure for cancer.
- Recruited and mentored new volunteers.