The role of Activating Transcription Factor 3 (ATF3) in chemotherapeutic induced cytotoxicity

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Abstract

Understanding the specific mechanisms regulating chemotherapeutic drug anti-cancer activities will uncover novel strategies to enhance the efficacy of these drugs in clinical settings.

Activating Transcription Factor 3 (ATF3) is a stress inducible gene whose expression has been associated with survival outcomes in cancer models. This study characterizes the chemotherapeutic drugs, cisplatin and Histone Deacetylase Inhibitor (HDACi), M344 as novel inducers of ATF3 expression. Cisplatin is a DNA damaging agent widely used in various tumour types including lung, head and neck, and ovarian carcinomas. The HDAC inhibitor, SAHA, has recently been approved as a single agent in the treatment of subcutaneous T-cell lymphoma and HDACis themselves show potential for synergistic anti-cancer effects when used in combination with established chemotherapeutic drugs, including cisplatin. This study evaluates the mechanisms by which cisplatin and HDACi induce ATF3, as well as the role ATF3 plays as a mediator of cisplatin-induced cytotoxicity and the enhanced cytotoxicity between HDACi and cisplatin in combination.

In this study, we demonstrate that cytotoxic doses of cisplatin and carboplatin consistently induced ATF3 expression in a panel of human tumour derived cell lines. Characterization of this induction revealed a p53, BRCA1, and integrated stress response (ISR) independent mechanism, all previously implicated in stress mediated ATF3 induction. Analysis of MAPKinase pathway involvement in ATF3 induction by cisplatin revealed a MAPKinase dependent mechanism. Cisplatin treatment, in combination with specific inhibitors to each MAPKinase pathway (JNK, ERK and p38) resulted in decreased ATF3 induction at the protein level. MAPKinase pathway inhibition led to decreased ATF3 mRNA expression and a reduction in the cytotoxic effects of cisplatin as measured by MTT cell viability assay. In A549 lung carcinoma cells, targeting ATF3 with specific shRNAs also attenuated the cytotoxic effects of cisplatin. Similarly, ATF3 -/- MEFs were shown to be less sensitive to cisplatin induced cytotoxicity as compared with ATF3+/+ MEFs. Taken together, we identified cisplatin as a MAPKinase pathway dependent inducer of ATF3 whose expression regulates in part cisplatin's cytotoxic effects.

Furthermore, we demonstrated that the HDAC inhibitor M344 was also an inducer of ATF3 expression at the protein and mRNA level in the same human derived cancer cell lines. Combination treatment with M344 and cisplatin lead to increased induction of ATF3 compared with cisplatin alone. Utilizing the MTT cell viability assay, M344 treatment was also shown to enhance the cytotoxic effects of cisplatin in these cancer cell lines. Unlike cisplatin, the mechanism of ATF3 induction by M344 was found to be independent of MAPKinase pathways. Utilizing ATF4 heterozygote (+/-) and knock out (-/-) mouse embryonic fibroblast (MEF) M334 induction of ATF3 was shown to depend on the presence of ATF4, a known regulator of ATF3 expression as part of the ISR pathway. HDACi treatment did not affect the level of histone acetylation associated with the ATF3 promoter as determined through Chromatin immunoprecipitation (ChIP) analysis, suggesting that ATF3 induction was not a direct effect of HDACi mediated histone acetylation. We also demonstrated that ATF3 regulates the enhanced cytotoxicity of M344 in combination with cisplatin as evidenced by attenuation of cytotoxicity in shRNAs targeting ATF3 expressing cells. This study identifies the pro-apoptotic factor, ATF3 as a novel target of M344, as well as a mediator of the co-operative effects of cisplatin and M344 induced tumour cell cytotoxicity.

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List of Abbreviations

Abbreviation	Definition
ABCC2	ATP- binding cassette sub-family C2
AdipoR2	adiponectin receptor 2
AP-1	Activating protein -1
APAF-1	apoptotic protease activating factor-1
ATF	Activating transcription Factor
ATP7B	copper transporting P-type adenosine triposphate
bZip	basic region leucine zipper
CA	catalytically active
c-Abl	v-abl Abelson murine leukaemia viral oncogene homologue
CDK	cyclin dependent kinase
ChIP	chromatin Immunoprecipitation
Cisplatin	Diamminedichloroplatinum (II)
CTR1	Copper transporter -1
DN	dominant negative
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EDTA	ethlyenediaminetetraacetic acid
eIF2α	eukaryotic initiation factor 2 α
EMSA	electrophoretic mobility shift analysis
EMT	epithelial-mesenchymal transition
ERCC1	excision repair cross-complementing -1
Erg-1	early growth response
GADD153/ CHOP	Growth arrest DNA damage inducible gene 153/C/EBP omologous protein
GADPH	glyceraldehydes 3-Phosphate dehydrogenase
GST	glutathione S-transferase
HAT	histone acetylase transferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HIF-1α	hypoxia-inducible factor
HRP	horseradish peroxidase
hsp27	heat shock protein 27
Id-1	inhibitor of different
IFN	interferon
IL	interleukin
IR	ionizing radiation
IRS2	insulin receptor substrate 2
ISR	integrated stress response
JNK	c-Jun N-terminal kinase
KLF-6	Kruppel-like factor
KSR1	kinase suppressor of Ras-1
LPS	lippopolysaccharide
MAPK	Mitogen-activated protein Kinase
MDM2	murine double minute
MEF	mouse embryonic fibroblast
MMR	mismatch repair
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide
NER	nucleotide excision repair
NK	natural killer
NSAID	non-steroidal anti-inflammatory
PARP	poly(ADP-ribose) Polymerase
PCR	polymerase chain reaction
PBS	phosphate buffer saline
PKC	protein kinase C
PTEN	phosphatase and tensin Homolog
RECK	reversion-inducing-cysteine-rich protein with kazal motifs
RNA	ribonucleic acid
ROS	reactive oxygen species
RPA	replication protein
RT-PCR	real time polymerase chain Reaction
SCCHN	small cell carcinoma head and neck
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel

electrophoresis

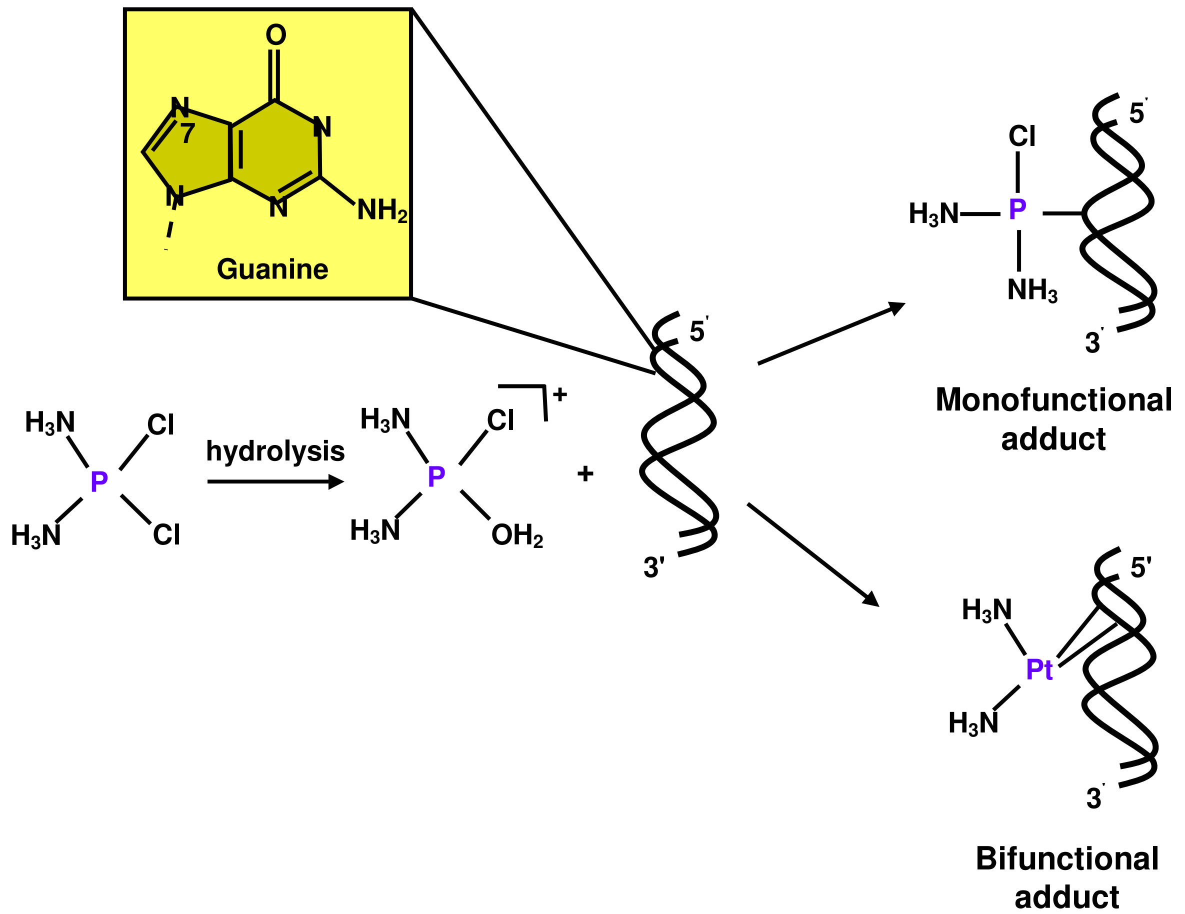
shRNA	short hairpin RNA
siRNA	small interference RNA
TBS	tris buffered saline
TFIIH	general transcription factor IIH
TGF-β	transforming growth factor-β
TIMP-1	tissue inhibitor of metalloproteinases-1
TLR-4	toll-like receptor-4
TNF-α	tumour necrosis factor
TRAIL	TNF- related apoptosis-inducing ligand
TTP	time to progression
UV	ultraviolet light
VEGF	vascular endothelial growth factor
WT	wild type
XIAP	x-linked inhibitor of apoptosis Protein
XPF	xerodermal pigmentosum complementation group F

Chapter 1: Introduction

1.1. Cisplatin and the mechanism of anti-cancer activity

cis-Diamminedichloroplatinum(II) (cisplatin) is among the most active anti-tumour agents used in human chemotherapy. Cisplatin and its derivative, carboplatin, are widely used agents in various tumour types including lung, head and neck, and ovarian carcinomas (1). Cisplatin and carboplatin are primarily considered as DNA-damaging anticancer drugs forming different types of bi-functional adducts in reaction with cellular DNA (1). The platinum based compounds are capable of diffusion across cell membranes where their positive charge of the metal platinum ion favours binding to the negatively charged nucleic acids of DNA (2). Cisplatin and carboplatin become activated intra-cellularly by the aquation of one or both chloride leaving groups, and subsequently covalently bind to nitrogen on position 7 of nucleic acid guanine, forming DNA adducts (Figure 1) (3). The major covalent bis-adduct that is formed following cisplatin treatment involves cross linking of adjacent guanines on the same strand of DNA, the intrastrand crosslink, and a minor adduct results from platinum binding to guanines on opposite DNA strands, the interstrand crosslink (1). Carboplatin is a less toxic compound as a result of the presence of a more stable leaving group than chloride which lowers toxicity, particularly the nephrotoxicity often associated with these agents (4). Efficacious treatments of carboplatin generally require up to 20 fold higher doses than cisplatin and are tolerated due to its decreased toxicity (5). It is widely accepted that the commencement of the anti-cancer effects of cisplatin involves DNA adduct formation which results in apoptotic cell death if the DNA damage cannot be adequately repaired (1). The final cellular outcome of DNA adduct formation is generally apoptotic cell death, thought to occur through halting of cellular processes such as replication

Figure 1. Mechanism of cisplatin – DNA adduct formation. Pathways for DNA adduct formation by cisplatin. The insert shows the structure of guanine and the position of N7, the major Pt binding site (Adapted from Kostova, 2006 (6)).



O
N
7
N
N
N
NH2
Guanine
H3N
Cl
P
H3N
Cl
hydrolysis
H3N
Cl
+
P
H3N
OH2
+
5'
3'
Cl
H3N
P
NH3
5'
3'
Monofunctional adduct
H3N
Pt
H3N
5'
3'
Bifunctional adduct

and transcription leading to prolonged G2 phase cell-cycle arrest, and deregulation of signal transduction pathways involved in growth, differentiation, and stress responses (1, 3).

1.2. Established mechanisms of cisplatin resistance

1.2.1. Mechanisms of reduced intracellular cisplatin and DNA binding

Cellular mechanisms of resistance to platinum-based chemotherapeutics are multi-factorial and contribute to the limitation of their use in clinical practice. They include molecular events inhibiting drug-DNA interaction, such as a reduction in cisplatin accumulation inside cancer cells or inactivation by thiol-containing species (7). Even though cisplatin can diffuse through the cellular membrane, the compounds are highly polar and therefore enter the cells relatively slowly (1). In addition to diffusion, cisplatin has been shown to enter tumour cells through plasma-membrane transporters, specifically, copper transporter-1 (CTR1) (8, 9). Downregulation of cell surface CTR1 expression has been reported in human ovarian cancer cell lines following cisplatin treatment whose mechanism occurs through the internalization of CTR1 from the plasma membrane by macropinocytosis, followed by proteasome-based degradation (10).

High affinity of platinum complexes for sulphur-containing biomolecules has also been implicated in cisplatin resistance. Although cisplatin's anti-cancer activity is related to it's ability to bind covalently to DNA ultimately leading to apoptotic outcomes, the percentage of cisplatin bound to DNA (5 –10%) is low compared with drug bound proteins (75–85%) (2). Preferred binding of platinum to thiol containing cellular compounds rather than bases of DNA is, therefore, a cause of resistance through sequestration of cisplatin from anti-cancer function (7, 11, 12).

Increased efflux of cisplatin has also been identified as a contributing factor to drug resistance. For example, overexpression of efflux protein copper-transporting P-type adenosine triphosphate (ATP7B), regulator of cellular copper levels, is associated with cisplatin resistance in vitro (13), and in vivo (14, 15). Likewise, reports have correlated exporter protein ATP-binding cassette, sub-family C 2 (ABCC2, also known as MRP2 or cMOAT) expression with cisplatin resistance in a variety of human derived cellular models (16, 17). Early reports had shown elevated levels of glutathione, thiol -containing tripeptide, could lead to platinum-drug resistance in ovarian cancer cell lines (18). It has been suggested that due to cisplatin's high affinity for conjugation with thiol-containing compounds, catalysed by glutathione *S*-transferases (GSTs), the resulting compound may be preferentially recognized and effluxed by the ATP-dependent glutathione *S*-conjugate export (GS-X) pump (ABCC2 or MRP2) thus resulting in reduced intracellular cisplatin leading to resistance (19, 20).

1.2.2. Mechanisms of resistance downstream of cisplatin and DNA conjugation

Other important mechanisms acting downstream to the initial reaction of cisplatin with DNA include an increase in adduct repair and a decrease in induction of apoptosis (7). Increased resistance to cisplatin has been shown to be correlated to DNA-adduct repair capacity (7). Nucleotide excision repair (NER) is the most common repair pathway of cisplatin DNA adducts (1). Increased expression of the NER endonuclease protein excision repair cross-complementing-1 (ERCC1), which functions through heterodimerization with xeroderma pigmentosum complementation group F (XPF) to make a 5´ incision into the DNA strand relative to the site of cisplatin-DNA adduct, has been correlated with resistance both in vitro and in vivo in an ovarian cancer model (21-23). Similarly, a role for the base

excision repair enzyme DNA polymerase-β in cisplatin resistance has been suggested. Studies have shown the overexpression of DNA polymerase-β in several cisplatin-resistant cell lines (24, 25), cell transfection with DNA polymerase-β genes resulting in increased cisplatin resistance (26), and observed increases in cisplatin efficacy following DNA polymerase-β antagonist treatment (27, 28).

Increased tolerance to cisplatin induced DNA damage has also been shown through loss of mismatch repair (MMR) DNA repair pathway. MMR pathway activity following platinum adduct formation is associated with apoptosis and increased cisplatin sensitivity (29, 30). This seemingly paradoxical observation has been postulated from the notion that cells that go through several unsuccessful repair cycles, likely mediated by the MMR pathway, lead to activation of the apoptotic response and therefore loss of MMR with respect to cisplatin–DNA adducts infers resistance (1, 7). Other reports suggest that the MMR pathway can induce apoptosis directly through upstream target activation of the tyrosine kinase v-abl Abelson murine leukaemia viral oncogene homologue (c-Abl) and pro-apoptotic factor p73 since loss of MMR proteins results in decreased activation and accumulation, respectively, following cisplatin treatment (31, 32).

Another important pathway of increased tolerance to cisplatin induced DNA damage leading to resistance is reduction in the apoptotic response. This reduction can result from either a loss of pro-apoptotic factors or an increase in anti-apoptotic factors. Cisplatin induced apoptosis can occur through both the extrinsic and intrinsic pathways and deficiency in apoptotic factors have been suggested to contribute to resistance for example, loss of tumour suppressor p53, caspases, and pro-apoptotic pathway Bcl-2 members has been shown to correlate with cisplatin resistance (2). P53 is an important tumour-suppressor protein that

is mutated in more than 50% of all human tumours (33). Mutations in p53 are commonly observed in exons 4–9 which disrupts the ability of the tumor suppressor to bind to DNA and transactivate p53-dependent genes (33-35). The p53 protein plays a role in a number of cellular processes which include cell cycle regulation, DNA repair and apoptosis (33-35). In response to DNA damage p53 is phosphorylated by the DNA damage response factor, ATM/ATR kinase (36). P53 inhibits cell proliferation by inducing either cell-cycle arrest, through transcriptional activation of cyclin-dependent kinase inhibitor p21WAF_1/Cip1 (p21), or apoptosis through transcriptional activation of pro-apototic BAX or repression of anti-apoptotic Bcl-2 protein in response to cellular stress (35, 37, 38). In addition, p53 interacts with components of the NER machinery, such as XPC (xeroderma pigmentosum, complementation group C), general transcription factor IIH (TFIIH) and replication protein A (RPA), indicating a role in DNA repair (39-41). Several studies demonstrate tumour cells expressing mutant p53 is a major mechanism in the reduction of the apoptotic response contributing to cisplatin resistance (3, 42-44). Cisplatin efficacy correlates positively with expression of wild-type p53 function in a National Cancer Institute (NCI) panel of 60 human tumour cell lines (45). Similarly, tumour cell lines lacking functional p53 are more resistant to cisplatin than cells that contain functional p53, and can be sensitized through reconstitution with wild-type p53 (46, 47). However there are reports which show no correlation between p53 status and response to cisplatin (2, 3, 44, 48, 49). Although p53's role as a tumour suppressor is well established, the discrepancy between p53 status and cisplatin cytotoxicity has been suggested to be dependent on several factors including tumor cell type, activation of specific signalling pathways, the presence of other genetic variations, and differences in p53 gene alterations (3, 44, 47).

Another factor in cisplatin-induced apoptosis is the non-receptor tyrosine kinase c-Abl. C-Abl is found in both the nucleus and cytoplasm, is activated upon DNA damage and has been implicated in cell cycle arrest and apoptosis through it's function as an activator of tumour suppressor targets, p53 and p73 (50). Several studies suggest a role for c-Abl tyrosine kinase in the activation of cisplatin induced apoptosis. For example, cisplatin induces c-Abl activation, and c-Abl-deficient cells are resistant to cisplatin-induced apoptosis (44, 51, 52). Reconstitution of functional c-Abl in Abl-deficient cells could restore cisplatin efficacy (53). Similarly, targeted c-Abl RNAi knockdown resulted in cisplatin resistance (54). C-Abl has been shown to be a mediator of p73 accumulation following cisplatin treatment and, therefore, been suggested to induce apoptosis through p73's known role in transcriptional activation of the pro-apoptotic factor, Bax (55, 56).

The key regulators of downstream apoptotic cascades cysteine protease, caspases, have also been implicated in cisplatin-induced apoptosis. Both apoptotic pathways, intrinsic, mediated by mitochondrial dysfunction, and extrinsic pathway, mediated by cell surface-death receptors (e.g., FAS), require the activation of initiator caspases (2, 57). Caspases-8 is activated through ligand death receptor binding leading to the aggregation of multiple procaspase-8 molecules and self-activation (2, 57). Activation of caspase-9 requires the formation of a large protein complex known as the apoptosome containing the apoptotic protease activating factor-1 (APAF-1) and cytochrome c as part of the intrinsic pathway (2, 57). Once activated, caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets ultimately leading to DNA fragmentation and cell death (2, 57). The caspase-dependent intrinsic pathway has been identified as a major pathway responsible for cisplatin induced cellular damage (2). Modulation of the anti-apoptotic factor, Bcl-2 and the pro-apoptotic factor, BAX ratio has been demonstrated in vitro to affect cisplatin induced

apoptosis (58, 59). Several studies also suggest a link between cisplatin toxicity and increased generation of reactive oxygen species (ROS) in the mitochondria (60, 61). For example, cisplatin has been demonstrated to induce expression of the enzyme cytochrome P450 which mediates ROS production and oxidative stress (62). Caspases involved in both intrinsic and extrinsic apoptotic pathways have been shown to play a role in cisplatin induced apoptosis as evidenced by loss of activation in resistant cells (31, 59, 63, 64). For example, loss of both caspase-9 and caspase-8 activation is associated with cisplatin resistance in the small cell carcinoma head and neck (SCCHN) cell line (65, 66). Caspase-3-deficient MCF-7 breast cancer cells have also been shown to be defective in apoptosis in response to cisplatin treatment, as determined by chromatin condensation, nuclear fragmentation, and release of cytochrome c from the mitochondria (64). Reconstitution with caspase-3 DNA in MCF-7 cells restores response to cisplatin mediated apoptosis (64).

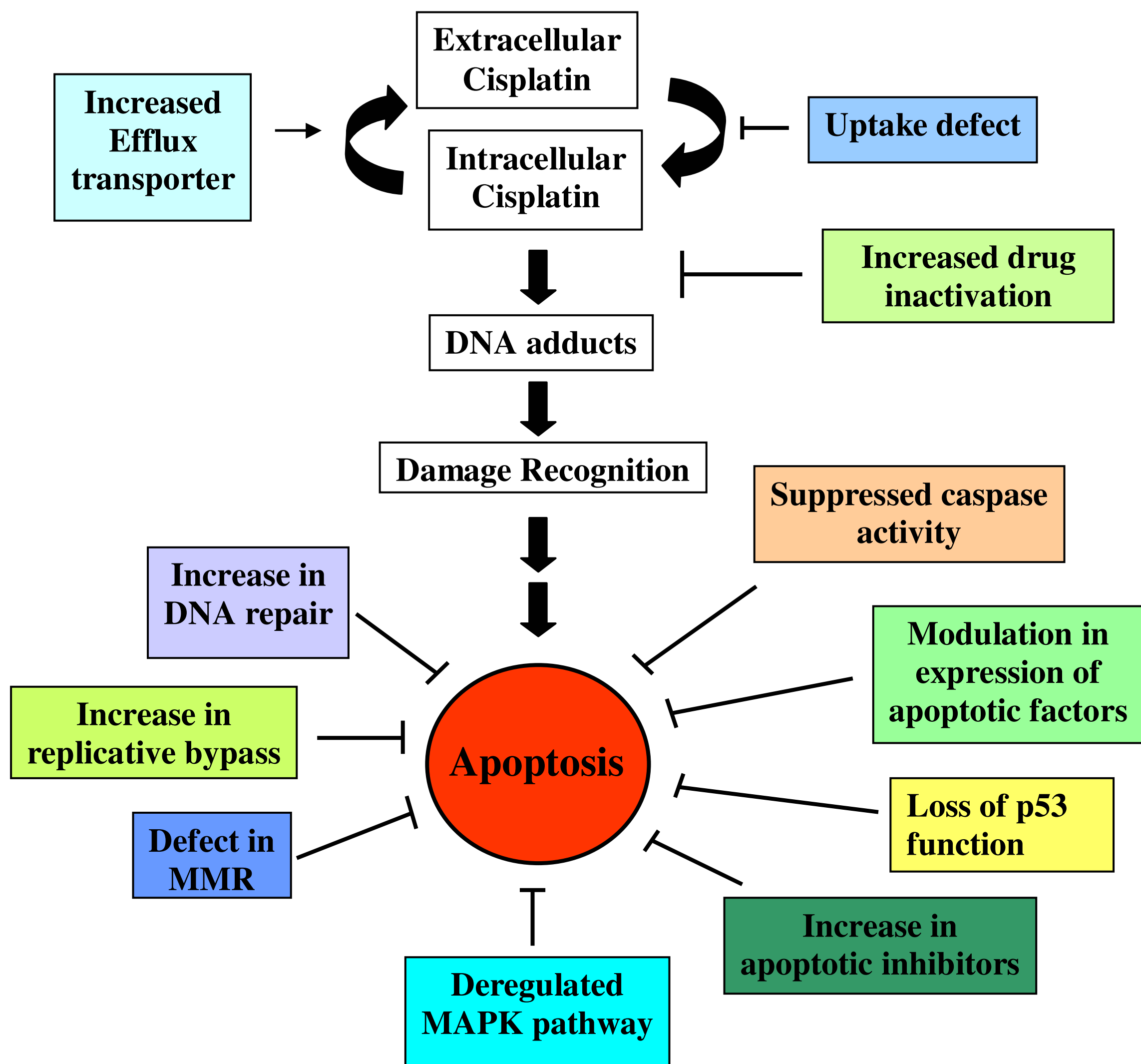
Not only are effectors of the apoptotic response implicated in mediating the cisplatin induced apoptotic response but so are apoptosis pathway inhibitors. For example, overexpression of X-linked inhibitor of apoptosis protein (XIAP) and (IAP-2) correlate with cisplatin resistance in some cell lines (67). Similarly, the anti-apoptotic factor survivin also correlates with cisplatin resistance; loss of which increased cisplatin induced apoptosis (67-69). Increased expression of the anti-apoptotic factors, Bcl-2 or Bcl-xl, with no affect on BAX expression, is shown to be associated with cisplatin resistance in an ovarian cancer patient model (70, 71). Hypoxia induced increase in Bcl-xl expression has been associated with cisplatin resistance (72), while antagonists to Bcl-2 or Bcl-xl have been shown to increase cisplatin efficacy (68, 72).

The Mitogen-activated protein kinase (MAPKinase) signalling cascades are activated by cisplatin and have been shown to be involved in drug resistance (44, 73-75). Members of

the MAPKinase family of proteins have important roles in transducing specific phosphorylation cascades activated by various extracellular stimuli (76, 77). Three mammalian MAPKinase subfamilies exist: ERK, JNK (also known as stress-activated protein kinase) and p38. The ERK pathway is mainly induced in response to growth factors and cytokines, whereas the JNK and p38 are activated in response to various stressors such as DNA damage (UV and IR), tumour necrosis factor, and hyperosmotic stress (76, 77). Reports have shown that activation of the p38 pathway by cisplatin has been exclusively correlated with pro-apoptotic outcomes in a number of cell lines, whereas activation of the JNK and ERK pathways are correlated with both anti- and pro- death outcomes (78). For example, inhibition of the p38 pathway with specific inhibitors and reduced activation of the p38 pathway has been shown to increase resistance to cisplatin (7, 73, 76). Interestingly, the p38 pathway has been shown to play a role in cisplatin induced apoptosis downstream of p53 (60, 79).

Acquired resistance and toxicities associated with chemotherapeutic treatment are major impediments inhibiting drug efficacy (Figure 2). Understanding the mechanisms regulating tumour cell cytotoxicity may uncover novel therapeutic strategies to enhance the efficacy of these platinum-based chemotherapeutics.

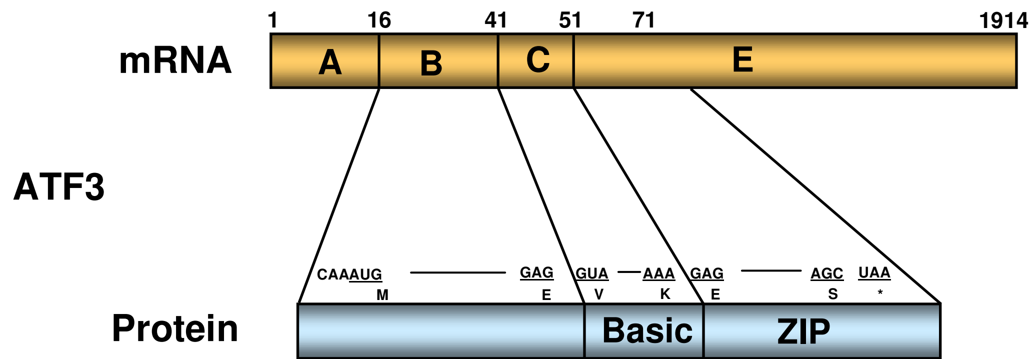
Figure 2. Mechanisms of cisplatin resistance. Schematic of the multifactorial pathways involved in cisplatin-resistance. (Adapted from Siddik, 2003 (3)).

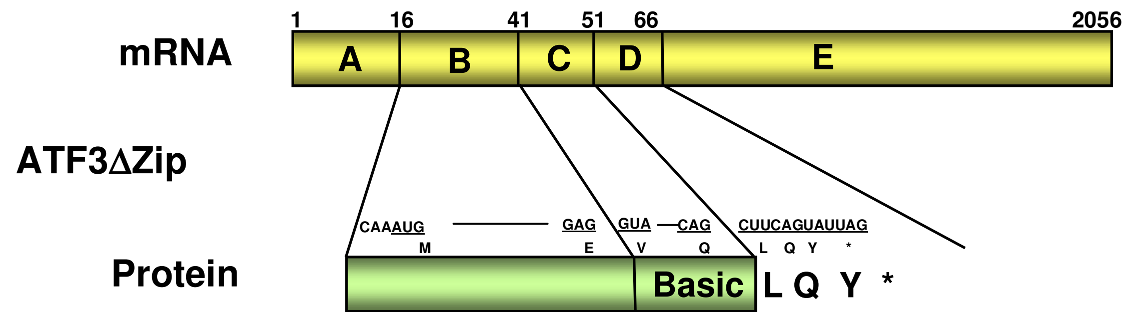

Extracellular Cisplatin
Intracellular Cisplatin
Increased Efflux transporter
Uptake defect
Increased drug inactivation
DNA adducts
Damage Recognition
Suppressed caspase activity
Increase in DNA repair
Modulation in expression of apoptotic factors
Increase in replicative bypass
Apoptosis
Loss of p53 function
Defect in MMR
Increase in apoptotic inhibitors
Deregulated MAPK pathway

1.3. Activating transcription Factor 3

Activating transcription factor 3 (ATF3) is a member of the basic region-leucine zipper (bZip) protein family originally identified for their ability to bind the cAMP responsive element (ATF/CRE) site (TGACGTCA) (80). These proteins form selective heterodimers with each other through their leucine zipper regions (80). The ATF3 gene, localized to chromosome 1q, transcribes ATF3 mRNA from four exons (A-E), distributed over 15 kilobases (kb), which translates an ~22 kilo Dalton (kDa) protein (81). The ATF3 mRNA contains exon A which encodes the 5'-untranslated region, exon B which encodes the initiation codon and the N-terminal, exon C which encodes primarily the basic region, and E which encodes the leucine zipper (ZIP) DNA binding domain and the 3'-untranslated region (Figure 3) (81). An alternatively spliced isoform has also been identified namely, ATF3Zip, which contains an additional exon between C and E introducing an in-frame termination codon that results in a truncated protein lacking the leucine zipper dimerization region and functional DNA binding motif at the C terminus (Figure 3) (81). This ATF3Zip isoform was shown through EMSA analysis, not to bind to DNA containing an ATF/CRE consensus sequence, whereas the longer wild type (wt) isoform ATF3 could specifically bind to ATF/CRE sites (82). Furthermore, this study showed that the wtATF3 isoform could act as a transcriptional repressor to promoters containing ATF/CRE consensus sites, where the ATF3Zip isoform, and a mutant lacking a functional DNA binding domain (ATF3 (1-100)), could stimulate transcription irrespective of the presence of an ATF binding sequence (82). From these observations the authors proposed the "co-factor model" which predicts that ATF3 functionally represses target promoters through interaction with inhibitory co-factors. Conversely, when ATF3 is functionally incapable of DNA binding, yet retains the ability to

Figure 3. ATF3 mRNA isoforms. Alternative ATF3 mRNA isoform translates a truncated protein. Exons in mRNAs are indicated by boxes labelled as A,B,C,D and E. Nucleotide numbers are indicated at the top. Functional domains of proteins are indicated by boxes. Amino acid numbers are at the bottom. (Adapted from Liang 1996 (81)).


1
16
41
51
71
1914
mRNA
A
B
C
E
ATF3
CAAAUG
GAG
GUA
AAA
GAG
AGC
UAA
M
E
V
K
E
S
*
Protein
Basic
ZIP


1
16
41
51
66
2056
mRNA
A
B
C
D
E
ATF3ΔZip
CAAAUG
GAG
GUA
CAG
CUUCAGUAUUAG
M
E
V
Q
L Q Y *
Protein
Basic
L Q Y *

interact with inhibitory co-factors, sequestration of these factors away from ATF site containing promoters results in transcriptional activation (82).

The 5'-flanking region of ATF3 has been studied and a number of transcriptional regulatory sites identified suggesting mechanistic regulation of the gene. The 5'-flanking region has revealed a consensus TATA element, promoter activity, and inducible transcription binding sites such as ATF/CRE, activating protein-1 (AP-1), and NF-KB (81). Two sites implicated in cell cycle regulation, Myc/Max and E2F, were also identified suggesting a potential cell-cycle dependent regulation (81). Indeed ATF3 has been shown as a regulator of cell cycle progression in cancer models (83).

Recently an alternative promoter (P1) has been identified ~44 kb upstream of the originally characterized promoter (P2) in the ATF3 gene (84). The ATF3 mRNA transcripts produced by the P1 promoter contains a novel 5'-UTR whose analysis has revealed a putative TATA box, and, not unlike that resulting from the originally identified promoter, a number of transcriptional binding sites including ATF/CRE, AP-1, p53, E2F, and NF-KB (84). Although the P1 directed transcript translates the identical protein as that of the P2, Miyazaki et al. identified a differential usage for the alternative promoter within the context of stress stimuli and cancer. For example, the P1 promoter was the major determinant responsible for serum-induction of ATF3, and the P1 transcript was dominantly expressed in constitutively overexpressing ATF3 Hodgkin Reed–Sternberg cell lines as compared with the P2 transcript (84).

ATF3 has also been identified as a member of the AP-1 transcription factors which consist of homodimers and heterodimers of the basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, v-Jun, JunB, JunD), Fos (c-Fos, v-Fos, FosB, Fra1, Fra2) and

the related activating transcription factor (ATF2, ATF3/LRF1, B-ATF) subfamilies (85). ATF3 has been shown to form heterodimers with ATF2, c-Jun, JunB, and JunD (86). These heterodimers can act as activators or repressors depending on promoter context, for example, ATF3/c-Jun and ATF3/JunD can activate promoters containing ATF/CRE sequences whereas ATF3/JunB can activate promoters containing ATF/CRE sites and repress promoters containing AP-1 sites (87). ATF3 homodimer has shown to act exclusively as a transcriptional repressor in studies with promoters containing ATF/CRE binding sites (86). Therefore, the role of ATF3 as a transcriptional regulator is dependent on interaction partners and promoter context. In general, the ATF3 homodimer is known to function as a transcriptional repressor, whereas when formed as heterodimers with Jun family members ATF3 functions as transcriptional activator (80).

1.4. ATF3 gene targets

1.4.1. ATF3's role in cell cycle progression and apoptosis

ATF3 expression has been linked to both cell cycle progression and apoptotic outcomes however, its role in these processes remains controversial given ATF3's demonstration as both an anti- and pro- apoptotic factor (83, 88). Clues as to ATF3's function as an apoptotic factor come from elucidation of ATF3 target genes (Table 1). For example, one of the first reported target genes of ATF3 was the growth arrest and DNA damage-inducible gene 153 (GADD153) also known as C/EBP homologous protein (CHOP), so named for it's ability to be induced following growth arrest and DNA damage signals (89, 90). GADD153/CHOP function has been determined as a member of the apoptotic branch of the ER stress response cascades (91). This pro-apoptotic role has been determined utilizing a

Table 1. ATF3 target genes.

Target gene	Cell type	ATF3 function	Cellular Function	Reference
GADD153/CHOP	Hela cell line	Repressor	Apoptosis (anti-apoptotic)	90
	MEFs	Activator	Apoptosis (pro-apoptotic)	98
ISR2	Beta cell line	Repressor	Apoptosis (pro-apoptotic)	102
Twist, FN-1, and Slug	Malignant breast cancer cell line MCF10A1a	Activator	Cell Mobility	88
Cyclin D1	Hepatoma cell line	Activator	Cell cycle progression	106
	MEFs	Repressor	Cell cycle arrest	83
Id-1	Human epithelial cell line	Repressor	Cell cycle arrest	107
Adiponectin	Adipocytes	Repressor	Obesity/insulin Resistance	108
AdipoR2	Hepatocytes	Repressor	Obesity/insulin Resistance	109
IL-6, IL-12b	Macrophage	Repressor	Innate immune Response	111
IL-12p40	HEK-293	Repressor	Innate immune Response	112
IL-4,-5, -13	Mouse CD4+ T cells	Repressor	Asthmatic Inflammatory Response	113
CCl4	Macrophages	Repressor	Innate immune Response	114
IFN-γ	NK cells	Repressor	Viral infection	115

CHOP knock out (KO) mouse model and downstream effectors mediating the apoptotic response have been suggested to involve those of the mitochondrial cell death pathways such as Bax, Bak and Bcl-2 (92-95). Wolfgang et al. identified GADD153/CHOP as a negatively regulated gene target of ATF3, which bound to the GADD153/CHOP promoter in an in vitro co-transfection HeLa cell model (90). The same group had previously shown a direct interaction between ATF3 and GADD153/CHOP which prevented ATF3 from binding to the ATF consensus site, demonstrating GADD153/CHOP as an inhibitor of ATF3's transcription regulatory function (96). Given these reports a negative regulation loop was proposed where under basal conditions GADD153/CHOP expression is high and inhibits ATF3 function, whereas ATF3, induced following stress insult, inhibits GADD153 gene expression thus protecting its functionality (90). ATF3 as a negative regulator of the pro-apoptotic factor GADD153/CHOP in the context of ER stress, or integrated stress response (ISR), would suggest ATF3 plays an anti-apoptotic role however, it has also been shown that ATF3 induction correlates with an increase in GADD153/CHOP expression (97, 98). Jiang et al. show that ATF3 expression is required for the nutrient stress induction of GADD153/CHOP acting through the eukaryotic initiation factor 2 alpha (eIF2-α), a member of the ISR pathway, as determined through contrasting ATF3 null and wt mouse embryonic fibroblasts (MEFs) (98). Taken together, ATF3 can function both as an anti- or pro-apoptotic factor through its role in direct gene modulation of the known ER stress induced pro-apoptotic factor, GADD153/CHOP.

Another target of ATF3 implicated in apoptosis is insulin receptor substrate 2 (IRS2), an intracellular second-messenger activated by insulin receptor-ligand binding shown to elicit signalling cascades involving insulin sensitivity and beta-cell survival and function

(99). IRS2 knock out genetic mice develop type 2 diabetes following insulin resistance and beta-cell failure (100, 101). Polymophisms in the IRS2 gene are rare and not considered a mediating factor in the development of type 2 diabetes and therefore an epigenetic silencing mechanism has been proposed (102). Li et al. show that through gain and loss of function approaches ATF3 downregulates IRS2 expression, binds specifically to the IRS2 promoter, and ATF3's induced repression of IRS2 is associated with a pro-apoptotic outcome in both in vitro and in vivo beta cell models (103).

In contrast, ATF3 plays an anti-cancer role in the aggressively malignant breast cancer cell line, MCF10A1a, where it was shown to promote cell migration (88). Yin et al. report that ATF3 ectopic expression correlates with increased expression of genes known to regulate cell mobility, such as Twist, fibronectin (FN-1) and Slug, which are suggested to be direct targets of ATF3 through promoter binding experiments (88). Of interest, ATF3's role as modulator of gene expression is not exclusively related to its transcriptional regulatory function. ATF3 has also been shown to directly interact with and promote the expression and protein stability of the tumour suppressor genes, p53 and p73, thus indirectly promoting their tumour suppressor activities (104, 105).

In relation to cell cycle progression two ATF3 target genes which function as cell cycle regulators have been identified. Cyclin D1, a cofactor to cyclin dependent kinase (CDK) which promotes cell cycle progression at the G1/S checkpoint, was first identified by Allen et al. as an ATF3 target gene (106). This group demonstrated an upregulation in cyclin D1 expression and promoter activity following ATF3 overexpression in a hepatoma cell line, as well as, specific binding between ATF3 and the cyclin D1 promoter correlating with increased cell cycle activity (106). In contrast, Lu et al. shows that serum starvation and Ras induced ATF3 expression repressed cyclin D1 expression correlating with cell cycle

arrest (83). Another cell cycle regulator gene identified as an ATF3 target is inhibitor of differentiation (Id-1), known for its role as a cell cycle exit antagonist (107). Kang et al. show ATF3, induced by transforming growth factor (TGF-β) and mediated by Smad3, represses Id-1 expression leading to cell cycle arrest in a human epithelial cell model (107). Taken together, these data demonstrate a role for ATF3 as a regulator of cell cycle activity given its function as a mediator of cell cycle regulator gene expression.

Adiponectin, an adipokine with insulin-sensing function whose decreased levels are linked to obesity and insulin resistance, has been reported to be negatively regulated by ATF3 in adipocytes (108). Likewise, a recent report shows the Adiponectin Receptor 2 (AdipoR2), which mediates insulin-sensing in the liver, is a repressible ATF3 target following ER stress stimulation (109). These reports implicate ATF3 in obesity-linked metabolic disease.

1.4.2. ATF3's role in immunity

A role for ATF3 in the innate immune response has been proposed through identification of a number of cytokines and chemokines, critical to the adaptive immune response, as direct target genes (110). Through a high-throughput systems biology approach using the Toll-like receptor 4 (TLR-4) agonist lipopolysaccharide (LPS) to stimulate macrophages, ATF3 was identified as an upregulated gene (111). This report, the first to implicate ATF3 in immunity function, identified cytokines Interleukin (IL)-6 and IL-12b as putative gene targets of ATF3 (111). ATF3 was shown to bind to the promoters of the two cytokines following LPS stimulation and ATF3's interaction with IL-6 and IL-12b promoters was shown to functionally repress their expression (111). Further in vivo analysis of

intraperitoneally (i.p.) LPS injected ATF3-null mice revealed elevated circulating levels of IL-6, IL-12b and tumour necrosis factor (TNF)-α as compared with wild type animals. ATF3-null mice were also shown to succumb to endotoxic shock induced death at a greater rate as compared with wild type mice (111). Taken together these data identified ATF3 as a negative regulator of cytokine/chemokine production as part of a negative feedback mechanism whose regulated expression is important in the development of autoimmune and inflammatory diseases, cancer and diabetes (110, 111). The same group goes on to propose a specific mechanism of cytokine silencing by ATF3 involving ATF3's association and recruitment of histone deacetylase (HDAC) to target promoters leading to histone deacetylation and chromatin condensation (111). Whitmore et al. broadened the spectrum on ATF3's role as a negative regulator of TLR pathways through usage of specific TLR ligands to TLR 2/6 heterodimer, 3, 5, 7, and 9 in a dendritic model in addition to macrophages (112). Whitmore et al. show that TLR-stimulated IL-12 levels were increased in myeloid dendritic cells from ATF3 KO mice compared with wild type animals, that ATF3 repressed the promoter of IL-12p40 as shown through reporter assay and ectopic ATF3 expression, and that ATF3 KO mice showed accelerated recovery from influenza virus stimulation compared with wild type mice (112).

Specific to an allergen-induced asthmatic model, ATF3 was shown to be upregulated following allergen insult and loss of ATF3 expression was associated with increased airway resistance, inflammatory cell accumulation, and increased expression of IL-4, 5 and 13 through ATF3 KO and WT mice lymphocyte compartment comparison (113). Notably ATF3 was shown to bind to the promoters of IL-4, 5 and 13 in lung derived CD4+ lymphocytes suggesting a role of ATF3 in mediating the asthmatic inflammatory response

(113). Consistent with ATF3's role as a negative regulator of the innate immune response, Khuu et al. show that chemokine CCL4 is negatively regulated by ATF3 under basal and pathogen stimulated conditions through comparison of KO versus WT ATF3 macrophages, and that ATF3 binds to the ATF/CRE site within the CCL4 promoter suggesting a direct regulatory mechanism (114). The cytokine, interferon (IFN-γ), known for it's role in the adaptive immune response, has also been shown to be a repression target of ATF3 in a Natural Killer (NK) cell model (115). This report shows that given IFN-γ's previous role as a protective agent in murine viral infection, ATF3 deficient mice showed beneficial viral clearance following murine viral challenge thus defining a role of ATF3 in viral immunity in addition to the previously discussed innate and allergy immune responses (115). Finally, a recent report characterizes a role for ATF3 in mediating Mast Cell (MC) function and maturation which demonstrates that ATF3 deficient MC are unresponsive to maturation signals and undergo increased apoptosis (116). This report suggests a broader role for ATF3 in adaptive immunity that extends past a cytokine/chemokine gene regulator and into immune cell function.

1.5. ATF3 is a stress inducible gene

A large body of evidence reveals that ATF3 is induced by a wide variety of stress causing agents which has lead to its description as a stress inducible gene (80, 86). ATF3 is induced during physiological stress such as liver regeneration (117), brain seizure (96), ischemia-reperfusion of the heart (118), and nerve damage (119, 120). ATF3 is also induced by a wide variety of both natural and synthetic stress stimuli such as, cytokines (121, 122), genotoxic agents such as ultraviolet light (UV) and ionizing radiation (IR) (123), JNK/SAPK

signalling pathway inducers anisomycin (124), cyclohexamide (125) and doxorubicin (126), amino acid starvation (98), microtubule binding agents such as taxol and colchicines (127), proteosome inhibitors such as lactacystin and MG132 (128), oxidative stressors such as hydrogen peroxide (129), and ER stressors such as thapsigargin (98). Our laboratory has extended this list to include the mevalonate pathway inhibitor, lovastatin (130), and chemotherapeutic drugs cisplatin (131) and histone deacetylase inhibitor (HDACi) (132).

1.6. ATF3 and cancer

A role for ATF3 in tumourigenesis has been implicated by reports that define ATF3 activity in cancer cellular pathways such as apoptosis, cell proliferation and tumourigenesis (110). The literature however, is divided as to whether to define ATF3 as an oncogene or tumour suppressor. A pro-apoptotic function is consistent with reports in which transgenic mouse models displaying tissue specific overexpression of ATF3 revealed conduction abnormalities and contractile dysfunction in the heart (133), liver dysfunction in the liver (129), and islet dysfunction with defects in glucose homeostasis in the pancreas (134, 135). In contrast, ATF3 overexpression protected rat hippocampal neuron cell death from kainic acid injection (136). ATF3 knock out (KO) mice display no lethality or obvious phenotype (134). This is consistent with the idea that ATF3 is a stress inducible gene which is not expressed or required under basal conditions.

ATF3 has also been shown to function as a regulator to cell proliferation and cell cycle progression. Gain of function experiments have shown anti-proliferation and cell cycle arrest functions of ATF3 in human cancer cells (123), and a loss of ATF3 function in a Ras transformed cell line model shows a higher growth rate and increased G1 to S phase transition efficiency (83). In contrast, mitogen ATF3 induction in hepatocytes has been

shown to increase cell cycle progression through cyclin D1 regulation (106), and overexpression of ATF3 in v-Jun transformed chicken embryo fibroblasts (CEFs) has been shown to enhance their growth rate (137).

ATF3 has also been implicated in cancer progression and malignancy. ATF3 has been shown to be play a dichotomous role in cell lines exhibiting varying degrees of malignancy. For example, ATF3 is pro-apoptotic in untransformed MCF10A mammary epithelial cells, but protects against cell death and enhances motility in the aggressively malignant MCF10CA1a cells (88). Evidence for a role as a tumour suppressor comes from the finding that anti-cancer agents such as progesterone (138) and cyclooxygenase inhibitors (139) have been shown to activate ATF3 expression. Overexpression of ATF3 in an ovarian cancer model suppressed cell growth, and reduced colony formation and cell invasiveness (138). Likewise, overexpression of ATF3 in the sense orientation in colorectoral cancer cells decreased focus formation *in vitro* and reduced the size of mouse tumour xenografts *in vivo* (139). ATF3 has also been shown to be a downstream target of the tumour suppressor p53 and ATF3 has been demonstrated as a p53 stabilizer by preventing its ubiquitination implicating ATF3 in it's tumour suppressor function (140). In contrast, ATF3 has been shown to be down-regulated by the tumour suppressor Drg-1 in prostate cancer cells and ATF3 overexpression has been shown to promote invasiveness *in vitro* and enhance metastasis *in vivo* in the prostate cancer cell model (141). Likewise, antisense knockdown of ATF3 in HT29 colon cancer cells resulted in inhibited cell attachment and invasiveness *in vitro* (142), and the constitutive expression of ATF3 in Hodgkin/Reed Stermberg cells promotes their viability (143). Taken together, the literature provides evidence for both pro- and anti-apoptotic activities, pro- and anti-proliferation functions, both cell cycle progression and arrest, and tumour suppressor and oncogenic functions. Conflicting observation of

ATF3 function has been suggested to stem from differences in the cellular model context and degree of cellular malignancy (83, 88).

1.7. Mechanism of ATF3 induction

Although it is known that ATF3 is induced by a wide variety of stress stimuli and the elucidation of down stream targets have shed light on ATF3's role in the regulation of cellular processes such as apoptosis, cell cycle, and immunity, less is known about the specific mechanisms of ATF3 induction. The p53 pathway, whose activity elicits cell cycle arrest through inhibition of the Rb/E2F cascade, has been implicated in ATF3 regulation. For example, it was reported that UV and MG132 induction of ATF3 was in part dependent on p53 expression, and p53 could increase ATF3 promoter activity and bind to the ATF3 promoter in a human cancer cell model (144). As mentioned earlier, ATF3 protein was found to bind to p53 stabilizing the protein by preventing its ubiquitin mediated degradation (140). These reports suggest a positive feedback loop promoting the tumour suppressor activities of p53 and thus a role for ATF3 in tumour suppressor function.

ATF3 has also been identified as a downstream effector of the ISR pathway. ATF3 is induced downstream of stress induced kinase eIF2α phosphorylation and ATF4 induction, and upstream of the ISR pro-apoptotic factor GADD153/CHOP (97, 98). Interestingly, a number of stress stimuli that activate the ISR pathway also induce ATF3 expression such as ER stressor thapsigargin (98), lovastatin (130), and amino acid starvation (98).

Other signalling pathways that have been implicated in the regulation of ATF3 expression are the MAPKinase cascades. An early report suggested the involvement of the JNK/SAPK pathway which observed that ATF3 could be induced following treatment with a

JNK/SAPK pathway agonist and that co-expression of ATF2 and c-Jun, two downstream effectors of the JNK/SAPK pathway, could activate the ATF3 promoter in a reporter assay (81). Since this first report, other groups have identified a role for MAPKinase pathways in ATF3 induction. For example, Cai et al. show that homocysteine induction of ATF3 in a vascular endothelial model was mediated through the JNK/SAPK pathway which was associated with ATF2 and c-Jun complex formation and activation of the ATF3 promoter (145). Similarly, Inoue et al. showed that TNF-α induced ATF3 was dependent on the JNK/SAPK pathway and the ERK pathway could antagonize this induction in an umbilical vein endothelial cell line (146). Lu et al. systematically evaluated the role of each MAPKinase pathway in mediating ATF3 induction by the protein synthesis inhibitor, anisomycin, and found the induction to be dependent on the p38 pathway and not JNK/SAPK or ERK pathways (124). Lu et al. also show that activation of the p38 pathway by a catalytically active kinase upstream of p38 and other various activators of the p38 pathway such as IL-1β, TNF-α, and hydrogen peroxide was sufficient to induce ATF3 (124). The same group identifies CREB, a downstream target of the p38 pathway, as a regulator of ATF3 induction using an ectopic expression approach (124). A very recent report shows the involvement of all three MAPKinase pathways in mediating the induction of ATF3 by the non-steroidal anti-inflammatory drug (NSAID) Tolfenamic acid, which is shown to be dependent upon ATF2 expression (147). The following thesis also describes results detailing the association between the MAPKinase pathways and the induction of ATF3 by the chemocytotoxic drug, cisplatin.

Other factors have been reported to play a role in the regulation of ATF3 expression. Tamura et al. report the induction of ATF3 by serum treatment is dependent on the

expression of the oncogene, c-myc, which they show binds to the ATF3 promoter in association with ATF2 and c-Jun and correlates with increased cell proliferation (148). Bandyopadhyay et al. report an inverse relationship between Drg-1 and ATF3 in a prostate cancer model where Drg-1 suppresses the activity of the ATF3 promoter and is associated with decreased metastasis (141). A recent report shows the tumour suppressor Kruppel-like factor (KLF-6) mediates the induction of ATF3 by binding to and activating its promoter which is correlated with increased apoptosis in prostate cancer cells (149). The DNA damage response factor BRCA1 has also been implicated in ATF3 regulation. Harkin et al. show increased ATF3 expression in a BRCA1 overexpression microarray analysis (150), and Fan et al. show increased ATF3 promoter activity following BRCA1 co-expression and reporter analysis (123). Finally, the tumour suppressor early growth response (Erg) 1 has been shown to positively regulate ATF3 in both colorectal cancer and gonadotrophin cell lines following induction by NSAID (139) and gonadotrophin releasing hormone (GnRH) receptor stimulation (151), respectively. Taken together, given the broad spectrum of signalling pathways and potential regulator factors governing ATF3 expression it is not surprising that ATF3 may be induced by such a vast range of stress stimuli.

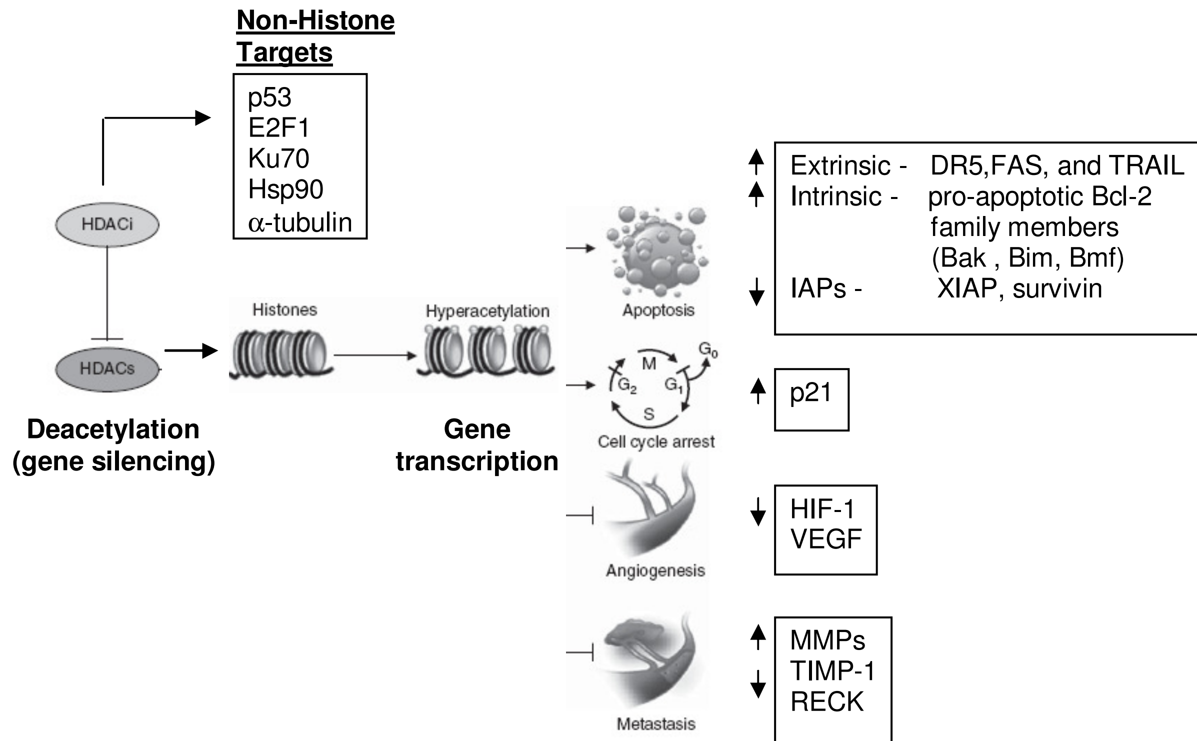
1.8. HDAC inhibition

1.8.1. HDAC inhibitor function and link to cancer

Alteration of gene expression plays a role in tumourigenesis and progression of cancer (152). Modulation of gene expression, for example, tumour suppressors, or oncogenes, are not exclusively due to mutations and can be manipulated through transcriptional regulation mechanisms which include DNA methylation and histone modification (153). In cancer cells, the balance between histone acetylation and deacetylation catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) is often disrupted (154). Altered expression of HDACs have been reported in tumour models for example, HDACs have been observed to be overexpressed in colon, breast, prostate, thyroid cervical and gastric cancers. Altered recruitment of HDACs to gene promoters mediated by chromosomal translocation mutations have also been reported in tumours (154, 155). Similarly, mutations in HAT proteins have been reported in leukemia (154, 155). HATs catalyze the addition of acetyl groups to histones which results in the decreased affinity between DNA and histones, promoting transcription factor binding to target promoter sites and thus active transcription. HDACs catalyze the removal of acetyl groups from histones resulting in chromatin condensation and transcriptional repression (153, 156, 157). HDAC inhibitors (HDACis) act to reverse this transcriptional silencing of genes, which include tumour suppressors (Figure 4) (153, 156, 157). HDAC function is not exclusive to histones, in that a number of non-histone protein substrates have been identified such as the tumour suppressor p53, whose deacetylation is associated with degradation (158).

Currently, 18 human HDACs have been identified. HDACs are classified into classes I through IV. Class I (HDAC1, 2, 3, and 8), IIa (HDAC4, 5, 7, and 9), class IIb

Figure 4. Histone Deacetylation; Mechanisms of Anti-Cancer Function. A schematic depicting the mechanistic action of histone deacetylase inhibitors as well as, the consequential gene modulation of factors that play a role in cellular processes dysregulated in cancer. Non-histone targets are also depicted. (Adapted from Ma, 2009 (153)).



Non-Histone Targets
p53
E2F1
Ku70
Hsp90
α-tubulin
HDACi
HDACs
Histones
Hyperacetylation
Deacetylation
(gene silencing)
Gene
transcription
Apoptosis
M
G0
G2
G1
S
Cell cycle arrest
Angiogenesis
Metastasis
Extrinsic - DR5,FAS, and TRAIL
Intrinsic - pro-apoptotic Bcl-2 family members (Bak , Bim, Bmf)
IAPs - XIAP, survivin
p21
HIF-1
VEGF
MMPs
TIMP-1
RECK

(HDAC6 and 10) and class IV (HDAC11) are zinc dependent deacetylases inhibited by broad spectrum HDAC inhibitors (152, 153). In contrast, class III (SIRT1 through 7) mediated deacetylases are dependent on NAD+ and are not inhibited by HDACis (152, 153). HDAC inhibitors have been developed as cancer therapeutics given their role in reversal of cancer associated epigenetic silencing. To date a minimum of 16 HDACi have been studied clinically at the phase I and II trial level with varying specificity among HDAC classes (153). The four major classes of HDACi are short-chain fatty acids (valproic acid and butyrate) which inhibit class I and IIa HDACs, hydroxamic acids (Vorinostat (SAHA), trichostatin A (TSA), LBH589, and PXD101) which inhibit class I, II and IV HDACs, benzamides (MS-275) which inhibit class I HDACs, and cyclic tetrapeptides (FK228) which specifically inhibit HDAC1 and 2 of class I (152, 153). Presently, only SAHA has been FDA approved for the treatment of subcutaneous T-cell lymphoma (159).

1.8.2. HDACi cellular mechanisms of anti-cancer activity

In general, HDAC inhibition is believed to elicit anti-cancer function through reports defining HDAC inhibitors as regulators to pathways affecting cancer development such as cell cycle progression, apoptosis, angiogenesis and metastasis (Figure 4). This HDACi regulation is thought to be achieved through its function as an inhibitor to histone deacetylation resulting in gene modulation which is consistent with cDNA microarray analysis showing an alteration of 7-10% of genes in various cancer cell lines following HDACi treatment (160-164). In addition to histone modification a number of non-histone protein substrates of HDACs have been reported which mediate cellular functions such as gene expression, differentiation and cell death (152, 153, 157). It is therefore likely that

acetylation modification of these targets may also contribute to HDAC inhibitor anti-cancer properties. Mechanisms of HDACi anti-cancer activity are discussed below.

1.8.2.1. Apoptotic Signalling Pathways

HDAC inhibitor induced cell death has been linked to the apoptotic extrinsic pathway which regulates apoptosis through ligand/death receptor binding mediated caspase activation. HDACi treatment has been shown to upregulate members of the extrinsic pathway in various transformed cell lines. For example, HDACi treatment enhanced the expression of death receptors DR-5 and Fas and their ligands FasL and TNF-related apoptosis-inducing ligand TRAIL, as well as, induced apoptosis mediated through TRAIL and Fas signalling pathways in a leukemia cell model (165). Similarly, HDACi induced caspase dependent apoptosis and increased expression of Fas and FasL in a neuroblastoma cell line (166), and the HDACi, LAQ824, induced the expression of DR-5 and reduced expression of the death receptor pathway inhibitor, FLIP-c in Jurkat human T-cell leukemia and SKW6.4B lymphoblast cell lines (167). Likewise, the HDACi sodium butyrate enhanced TRAIL mediated apoptosis and upregulated DR-5 expression in a colon cancer cell line (168). Taken together these reports indicate a role for HDAC inhibition in the regulation of cell death outcomes through the extrinsic apoptotic pathway.

Many reports also implicate HDAC inhibitors in intrinsic pathway mediated apoptosis. The intrinsic pathway acts through Bcl-2 family member mediated release of the mitochondrial protein, cytochrome c, resulting in apoptosome formation and caspase activation (57). HDACi treatment has been shown to modulate the expression of intrinsic pathway regulators. For example, Xu et al. reports HDACi treatment results in an up-regulation of pro-apoptotic intrinsic pathway factors Bak, Bax, Bim, Bmf and Bik, a down-

regulation of anti-apoptotic factors XIAP and survivin, and inhibition of the anti-apoptotic factor Bcl-2 correlating with a decrease in HDACi induced cell death in a prostate cancer cell model (169). Rosato et al. show that combinational treatment with HDACi and TRAIL could enhance mitochondrial damage, caspase activation and apoptosis (170). Dual treatment also resulted in Bid activation, Bax translocation, ectopic expression of anti-apoptotic factors Bcl-2 and Bcl-XL, and the attenuation of HDACi induced apoptosis implicating HDACi in mitochondrial dependent apoptosis (170). Ruefli et al. also show HDACi mitochondrial mediated cell death through observations of increased cytochrome c release and ROS production, and Bid cleavage (171). Similar to Rosato et al., Ruefli et al. also report that the overexpression of Bcl-2 caused a decrease in HDACi mediated cell death in a leukemia cell line model (171). Zhang et al., show that HDACi treatment results in increased mitochondrial membrane permeability which could be augmented by Bcl-2 expression (172). They also observe an increase in pro-apoptotic factors Bim, Bax and Bak, and a decrease in anti-apoptotic factors XIAP, Bcl-XL and Mcl-1 in a melanoma cell line (172). These data demonstrate an involvement of HDACi in intrinsic pathway activation and factor modulation correlating with enhanced cell death.

The anti-cancer activity of HDACi has also been correlated with the generation of ROS. Studies have shown that HDACi treatment leads to increased ROS accumulation in transformed cell models (169, 171, 173). Ruefli et al. report a block in HDACi induced cell death when treated in conjunction with antioxidants in a leukemia cell line suggesting a role for the observed increase in ROS in HDACi induced apoptosis (171). Mechanistically, it has been proposed that HDACi mediated ROS accumulation is correlated to it's ability to modulate factors that regulate ROS production (169, 174). Thioredoxin (Trx) functions as a hydrogen donor and scavenger of ROS, and thioredoxin binding protein-2 (TBP-2) binds to

and inactivates Trx (169, 174). Studies show that HDACi treatment upregulates TBP-2 and downregulates thioredoxin expression in transformed cell lines (169, 174). Similarly, Ungerstedt et al. reports that siRNA knockdown of Trx results in inhibition of cell growth and increases sensitivity to HDACi induced cell death in transformed lung fibroblasts (173).

1.8.2.2. Cell Cycle Regulation

HDAC inhibitors are known for their cell cycle arrest function (153, 157). In general, cell cycle arrest induction by HDACis is believed to be related to their direct function as inhibitors of histone deacetylation which specifically enhance the expression of the cell cycle suppressor gene p21 (175, 176). P21 is known for its cell cycle regulatory role as an inhibitor of the cyclin dependent kinase complexes CDK2/cyclin E and CDK4/6/cyclin D which govern G1 to S phase progression and the cdc2/cyclin B complex which mediates G2 to M phase advancement (177). HDAC inhibition has been correlated with increased p21 expression and enhanced acetylation of histones associated with the p21 promoter (175, 176). Gui et al. report a marked decrease in HDAC1 and Myc and an increase in RNA polymerase II association with the p21 promoter following HDACi treatment in a human mylenoma cell line (175). Unlike p21, the promoter of p27, another CDK inhibitor, was not found to associate with increased acetylation (175), however studies have shown an upregulation of p27 expression in HDACi treated transformed cell lines (178, 179). Others report that HDAC inhibitors can lead to downregulation of cyclin expression (180-182). These data mechanistically link HDACi to cell cycle function and ultimately anti-cancer activity.

1.8.2.3. Angiogenesis

Angiogenesis, the process of blood-vessel formation that plays an important role in tumour growth and metastasis, has been implicated in HDACi induced anti-cancer function. HDACis have shown anti-angiogenic properties which are believed to be related to their ability to augment gene expression of factors that mediate angiogenesis such as hypoxia-inducible factor (HIF-1α) and vascular endothelial growth factor (VEGF) (183-185). HDACi has been reported to downregulate expression as well as activity of HIF-1α, which under hypoxic conditions functions as a transcription factor to induce the expression of factors which propagate the angiogenesis cascade (184). Inhibition of angiogenesis by HDACis has also been shown to be related to their ability to downregulate the expression of VEGF, a growth factor which binds to its receptor on endothelial cells of neighbouring blood vessel initiating cell proliferation and migration (185, 186). HDACis therefore may provide potential therapy against tumour associated angiogenesis.

1.8.2.4. Metastasis

HDACis have also shown anti-metastatic function thought to be related to their ability to augment genes involved in the metastasis pathway (153). For example, demonstrated in an *in vivo* prostate cancer tumor model, HDACi treatment resulted in reduced migration of prostate tumour cells, inhibition of metastatic lung lesions and an increase in the metastasis suppressor, tissue inhibitor of metalloproteinases-1 (TIMP-1), expression (187). Liu et al. also show induction of metastasis suppressor reversion-inducing-cysteine-rich protein with kazal motifs (RECK) by HDACi leading to inhibition of pro-metastasis factor, matrix metalloproteinase 2 (MMP-2) activity and suppressed invasiveness

in a lung cancer cell model (188). Similarly, Joseph et al. report that HDACi treatment downregulates expression of MMPs, integrins, and collagens following cDNA microarray analysis in a lung carcinoma cell line (189). These data provides evidence for an anti-metastasis role for HDACis in cancer models linked to alterations in gene expression.

1.8.2.5. Non-Histone protein substrates

Not only do HDACs deacetylate histones they also target non-histone proteins such as transcription factors and various cytoplasmic proteins with diverse cellular functions. Therefore, HDACi anti-cancer activity is not limited to histone-mediated transcriptional regulation, but may also be influenced by the anti-cancer functions of non-histone targets. For example, the classic tumour suppressor p53 has been shown as a target of deacetylation by HDAC1 (158). Ito et al. show that p53 undergoes deacetylation by HDAC1 in complex with p53's classical negative regulator murine double minute (MDM2) (158). Ito et al. also show that loss of HDAC1 function is associated with increased p53 acetylation, expression, and increased expression of downstream p53 targets such as p21 (158). This data suggests that acetylation of p53 stabilizes the protein and that HDAC1-MDM2 associated complexes act to destabilize p53 through deacetylation and degradation (158). HDAC inhibition therefore promotes p53 stability and function of the tumour suppressor. HDAC function has also been shown to directly influence acetylation of the transcription factor E2F which when activated, controls cell cycle progression at the G1/S checkpoint (190). Reports show E2F acetylation is associated with increased DNA binding capacity, increased expression and increased transactivation potential and HDAC mediated deacetylation negatively regulates E2F activity (190, 191). In terms of anti-cancer function related to E2F activation, E2F/HDAC associated complex has been shown to negatively regulate expression of tumour

suppressor Ras homologue member I (ARHI) and HDACi was shown to increase ARHI promoter activity which correlated with increased E2F acetylation (192). Both transcription factor targets provide anti-cancer potential through inhibition of HDAC function and therefore may play a mechanistic role in HDACi anti-cancer properties.

Other cytoplasmic HDAC targets have been identified with diverse cellular functions such as DNA repair (Ku70), chaperones (Hsp90) and structure integrity (α–tubulin) (157). Deacetylation of the DNA repair factor Ku70 by HDAC has been shown to increase DNA repair activity and HDACi treatment results in reduced Ku70 DNA binding affinity, and DNA repair capacity in cancer cells (193). Loss of DNA repair capacity following chemotherapeutic anti-cancer agent treatment is advantageous in a cancer therapeutic model given the high capacity for drug resistance. The chaperone protein Hsp90 is a target of HDAC deacetylation, commonly known for its role in protein folding/degradation (194, 195). HDAC inhibition and increased Hsp90 acetylation is associated with impaired function leading to blockages in downstream cell growth signalling cascades (194, 195). HDACi mediated acetylation of α-tubulin, another substrate of HDAC deacetylation a and structural protein functioning in cell mobility, has been correlated with decreased mobility in carcinoma cells (196). These data suggest an anti-cancer role for HDACi independent of histone mediated transcriptional regulation.

1.9. HDAC inhibitors enhance the anti-cancer activity of established anti-cancer agents

Pre-clinical and clinical studies have demonstrated that HDAC inhibitors can enhance the anticancer activity of a variety of epigenetic as well as chemotherapeutic agents (152, 153). The list of anti-cancer agents HDAC inhibitors have been shown to act in synergy

include DNA damaging agents (carbo- and cis-platin and γ-irradiation), microtubule destabilizing agents (Paclitaxel), topoisomerase II inhibitors (doxorubicin and epirubicin), DNA demethylating agents (hydralazine, azacitidine), anti-metabolites (gemcitabine), death receptor agonists (TRAIL), proteosome inhibitors (bortezomib), and kinase inhibitors (LY294002) (152, 157). In general, HDAC inhibitor's broad capacity for synergy is believed to be related to an ability to lower the threshold for apoptosis given HDACi's role as a mediator of genes involved in pathways dysregulated in cancer development such as cell cycle control, apoptosis, angiogenesis and metastasis (153). Specific mechanisms mediating synergism between HDAC inhibitors and different classes of anti-cancer agents have been proposed. For example, enhanced cytotoxic effects of HDAC inhibitors in association with DNA damaging agents such as γ-irradiation, may be related to their function as inhibitors to histone deacetylation resulting in loosely condensed chromatin which would physically promote increased access of DNA damaging agents to DNA (153, 197). Another postulation is that HDACis having been shown to influence the accumulation of DNA damaging molecules such as ROS, as well as impair the function of DNA repair factor Ku70, may function as a positive regulator of the DNA damage response when combined with DNA damaging agents (197). The synergism observed between HDACis and proteosome inhibitors has been proposed to be related to HDACi's known role in the inactivation of chaperone protein Hsp90 (197). Blockage in proteosomal degradation is potentially toxic as misfolded protein and aggregates accumulate. Given HDACis role in inactivation of Hsp90 the balance towards cell survival may be shifted towards cell death when combined with proteosome inhibitor treatment (197). Lastly, given HDACis role as positive regulators of apoptotic factors such as death receptors and their ligands, synergism with death receptor

pathway agonists would presumably enhance their apoptotic effect (152, 197). Thus, the broad spectrum of reported synergism between HDACis and established anti-cancer agents is not surprising given their great potential for regulation of mechanisms involved in anti-cancer function.

2.0. Rationale and Hypothesis

The platinum-based chemotherapeutic drug, cisplatin is used extensively in cancer therapeutic regimens for the treatment of a range of cancers including lung, ovarian and breast. Although believed to be driven by the induction of DNA damage, the mechanisms downstream of cisplatin induced DNA adduct formation leading to tumour cell cytotoxicity are poorly defined. Roles for the tumour suppressor p53 and the cell growth and differentiation regulating MAPKinase pathways in mediating cisplatin induced cytotoxicity have been demonstrated.

The HDAC inhibitor has been recently approved as a single agent chemodrug therapy for subcutaneous T-cell lymphoma. The role of HDAC inhibitors in inducing anti-cancer responses are attributed to their broad capacity for gene regulation through their function as inhibitors to deacetylation for both histones, thus facilitating active transcription, and non-histone substrates with varying anti-cancer functions, such as p53. HDAC inhibitors have been shown to synergistically enhance the anti-cancer effects of established chemotherapeutic drugs, including cisplatin.

ATF3 is a stress inducible gene whose role in tumourigenesis has been demonstrated as both a tumour suppressor and an oncogene that is cell type and context dependent. Proposed pathways mediating ATF3 induction include p53, MAPKinase and the ISR.

Hypothesis

Since various stress pathways that induce ATF3 expression regulate cell survival (MAPKinase, p53 and ISR) as well as the anti-cancer effects of cisplatin and HDACi, ATF3 plays an important role in regulating chemotherapeutic drug induced cytotoxicity by either cisplatin alone or in combination with HDACi.

2.0.1. Objectives:

Chapter 2

1) Characterize ATF3 induction by cisplatin in a human derived cancer cell line model.

2) Determine the mechanism of ATF3 induction by cisplatin.

3) Determine the role of ATF3 in cisplatin induced cytotoxicity.

Chapter 3

4) Characterize ATF3 induction by HDACi and HDACi in combination with cisplatin.

5) Determine the mechanism of ATF3 induction by HDACi.

6) Determine the role of ATF3 in the enhanced cytotoxic effects of HDACi and cisplatin combinational treatment.

Chapter 2: Results

Cisplatin induces cytotoxicity via the mitogen-activated protein kinase pathways and Activating Transcription Factor 3

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Key words: activating transcription factor 3, cisplatin, mitogen-activated protein kinases

Running Title: ATF3 regulates cisplatin cytotoxicity

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Contribution of authors:

This manuscript was written by Carly St. Germain and edited by Dr. Jim Dimtroulakos. All experiments were performed by Carly St. Germain with the exception of Figure 1A in which Dr. Laurie Ma assisted with MTT assays, Figure 2B immunocytochemistry conducted by technical assistant Anna O'Brien, and Figure 6C and D MTT assays conducted by Kyla Garbuio.

This thesis is presented in a paper-based format where references displayed at the end of the chapter are specific to this chapter.

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Abstract

The mechanisms underlying the pro-apoptotic effect of the chemotherapeutic agent, cisplatin, are largely undefined. Understanding the mechanisms regulating cisplatin cytotoxicity may uncover strategies to enhance the efficacy of this important therapeutic agent. This study evaluates the role of Activating Transcription Factor 3 (ATF3) as a mediator of cisplatin-induced cytotoxicity. Cytotoxic doses of cisplatin and carboplatin treatments consistently induced ATF3 expression in five tumour derived cell lines. Characterization of this induction revealed a p53, BRCA1, and integrated stress response (ISR), independent mechanism, all previously implicated in stress mediated ATF3 induction. Analysis of MAPKinase pathway involvement in ATF3 induction by cisplatin revealed a MAPKinase dependent mechanism. Cisplatin treatment combined with specific inhibitors to each MAPKinase pathway (JNK, ERK and p38), resulted in decreased ATF3 induction at the protein level. MAPKinase pathway inhibition led to decreased ATF3 mRNA expression and a reduction in the cytotoxic effects of cisplatin as measured by MTT cell viability assay. In A549 lung carcinoma cells, targeting ATF3 with specific shRNAs also attenuated the cytotoxic effects of cisplatin. Similarly, ATF3 -/- MEFs were shown to be less sensitive to cisplatin induced cytotoxicity as compared with ATF3+/+ MEFs. This study identifies cisplatin as a MAPKinase pathway dependent inducer of ATF3, whose expression influences cisplatin's cytotoxic effects.

Introduction

cis-Diamminedichloroplatinum(II) (cisplatin) is among the most active anti-tumour agent used in human chemotherapy. Cisplatin and its derivative, carboplatin, are widely used agents in various tumour types including lung and ovarian cancers [1.2]. Acquired resistance and toxicities associated with treatment are major impediments inhibiting their efficacy [2.2]. Understanding the mechanisms regulating tumour cell cytotoxicity may uncover novel therapeutic strategies to enhance the efficacy of these platinum-based chemotherapeutics. Cisplatin and carboplatin are primarily considered as DNA-damaging anticancer drugs forming different types of bi-functional adducts in reaction with cellular DNA [1.2]. Cisplatin and carboplatin become activated intra-cellularly by the aquation of one of two chloride leaving groups, and subsequently covalently bind to DNA, forming DNA adducts [3.2]. Carboplatin is a less toxic compound with a more stable leaving group than chloride which lowers toxicity, and reduces nephrotoxicity [4.2]. Efficacious treatments of carboplatin generally require up to 20 fold higher doses than cisplatin and are tolerated due to its decreased toxicity [5.2]. The final cellular outcome of DNA adduct formation is generally apoptotic cell death, thought to occur through halting of cellular processes such as replication and transcription leading to prolonged G2 phase cell-cycle arrest, and deregulation of signal transduction pathways involved in growth, differentiation, and stress responses [1.2, 3.2]. Cellular mechanisms of resistance to platinum-based chemotherapeutics are multi-factorial and contribute to severe limitation in their use in clinical practice. They include molecular events inhibiting the drug-DNA interaction, such as a reduction in cisplatin accumulation inside cancer cells or inactivation by thiol-containing species [2.2]. Other important mechanisms acting downstream to the initial reaction of cisplatin with DNA, include an increase in adduct repair and a decrease in induction of apoptosis [2.2]. Although

DNA is the primary target of cisplatin and carboplatin activity, there still remain gaps in our understanding of the process that translates cisplatin induced DNA damage into its therapeutically beneficial process of apoptosis. Two significant cellular pathways have been demonstrated to play key roles in platin-induced apoptosis/cytotoxicity, the mitogen activated protein kinase cascades (MAPKinase) and the tumor suppressor p53 [6.2, 7.2]. An understanding of the mode of action is indeed desirable in refining therapeutic approaches that further enhance the anti-tumour activity of platinum based chemotherapeutics.

Activating transcription factor 3 (ATF3) is a member of the basic region-leucine zipper proteins originally identified for their ability to bind the cAMP responsive element (ATF/CRE) site (TGACGTCA) [8.2]. While ATF3 mRNA and protein levels are not detectable under basal conditions in most cells, a large body of evidence shows that ATF3 is induced by a wide variety of stress causing agents including hypoxia, metabolic stress and DNA damage [9.2]. ATF3 is also induced in times of physiological stress such as liver regeneration [10.2], brain seizure [11.2], ischemia-reperfusion of the heart [12.2] nerve damage [13.2, 14.2] and UV damage where it plays a role in maintaining genomic integrity [15.2]. ATF3 has been demonstrated to play a role in apoptosis and proliferation, two cellular processes critical for cancer progression [16.2-19.2]. ATF3 can either promote or suppress these processes. For example, over expression of ATF3 in the sense orientation in colorectal cancer cells led to decreased focus formation *in vitro* and reduced the size of mouse tumour xenografts *in vivo* [16.2]. Divergence in function of ATF3 between a pro-and anti-apoptotic factor in cancer models is likely dependent on both the cellular model and the state of malignancy [19.2, 20.2]. ATF3 is also a member of the Activating Protein–1 (AP-1) transcription factors which consist of homodimers and heterodimers of the basic region-leucine zipper proteins that belong to the Jun (c-Jun, v-Jun, JunB, JunD), Fos (c-Fos, v-Fos,

FosB, Fra1, Fra2) and the related activating transcription factor (ATF2, ATF3/LRF1, B-ATF) subfamilies [21.2]. Activation of ATF3 by a wide array of stress signalling pathways have been demonstrated including DNA repair pathway components p53 [22.2, 23.2] and potentially BRCA1 [17.2, 24.2], the integrated stress response (ISR) that is principally activated by hypoxia and metabolic stress [25.2], and the stress induced MAPKinase cascades (SAPK/JNK, and p38) [26.2, 27.2]. Of interest, p53 and the p38 MAPKinase pathway have also been shown to play roles in regulating cisplatin-induced cytotoxicity [28.2, 29.2].

In this study, we evaluated the potential of cisplatin to induce ATF3 and determined the pathway regulating this induction. Furthermore, we determined the role of ATF3 as a mediator of the cytotoxic effects of cisplatin.

Material and Methods

Tissue Culture. The A549, PC3, HCC1937 and MCF-7 cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cell lines SKOV-3 and A2780-cp were kindly provided by Dr. Barbara Vanderhyden, Ottawa Hospital Research Institute, Ottawa, Canada. The MEFs used in this study were derived from wild type and knockout mice from ATF3, ATF4 and ATF2 models kindly provided by D. Park (University of Ottawa, Ottawa, Ontario) and L. Glimcher (Harvard Medical School, Boston, MA). All cell lines were maintained in DMEM (Media Services, Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (FBS; Medicorp, Montreal, Canada) and 100 units penicillin and 100μg streptomycin (GIBCO, Burlington, ON) / ml of media. Cells were exposed to cisplatin, carboplatin and taxol (provided by the pharmacy at the Ottawa Hospital Regional Cancer Centre, Ottawa) alone or in combination with the p38 inhibitor SB203580 (Calbiochem, Gibbstown, NJ), JNK inhibitor, JNK inhibitor II (SP600125) (Calbiochem) or ERK inhibitor UO126 (Calbiochem) diluted in DMSO. Adenovirus p53wt and LacZ control were kindly provided by Dr. Bruce McKay (Ottawa Hospital Research Institute, Ottawa, Canada).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay. In a 96-well flat-bottomed plate (Nunc, Naperville, IL) 5,000 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 indicated drugs and incubated for 48 h at 37°C. Following treatment, 42 μL of a 5 mg/mL solution in PBS of the MTT tetrazolium substrate (Sigma) was added to each well and incubated for ~ 20 min at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 82 μL of a 0.01 mol/L HCl/10% SDS (Sigma)

solution, and allowed to further incubate at 37°C overnight. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies (West Sussex, United Kingdom) at 570 nm to determine the absorbance of the samples.

Flow Cytometry. Cells were plated at 1 X 10^6 / 10 cm dish and allowed to grow overnight and subsequently treated with cisplatin for 48 h. Single cell suspensions were labelled with 50 μg/ml propidium iodide (Sigma) and approximately 10^6 cells in 1 ml analyzed by flow cytometry. Ten thousand cells were evaluated and the percentage of cells in sub -2N phase determined using the Modfit LT program (VeritySoftware House, Topsham, Maine).

Immunocytochemistry. MCF-7 and PC3 cells grown to 50% confluence were seeded on 2 x 15cm plates/treatment/block with no treatment or with cisplatin (8μg/ml) for 24 h. Cells were washed twice in, PBS, harvested in 10 ml PBS/ plate and combined with 20 ml of 20% Neutral Buffered Formalin (Sigma). Cells were fixed at 4°C for 1 h spun down at 1600rpm, for 10min at 4° C and cells were washed once in PBS. Formalin-fixed cells were paraffin embedded, cut into 5 um sections and allowed to dry at room temperature overnight. Sections were deparaffinized by washing in toluene (3 X 5 min) followed by absolute alcohol (2 X 1 min). Sections were washed with water (5 min) followed by Tris Buffered Saline (TBS) and loaded on the intellliPAT FLX automated slide stainer. Automated slide stainer was programmed with the following treatments: 3% H_2O_2 in TBS for 10 min, rinsed in TBS for 5 min, blocked with universal blocking agent Background Sniper (Biocare Medical; Brampton, ON, Canada) for 20 min at room temperature, incubated with ATF3 antibody (1:200 dilution in DaVinci universal diluent (Biocare Medical)) for 1 h at room temperature, rinsed with TBS for 5 min, incubated with universal mouse probe (Biocare

Medical Mach 4 universal polymer detection kit) for 5 min, rinsed with TBS for 5 min, incubated with Rabbit HRP Polymere (Biocare Medical Mach 4 universal polymer detection kit) for 10 min at room temperature, rinsed with TBS, and developed for 5min with DAB RTU (Biocare Medical Betazoid DAB chromatogen kit) and rinsed with water. Slides were counterstained in hematoxylin for 1 min, washed in running water, 0.2% HCl in 70% alcohol for 5 dips, 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.

Transfection. MCF-7 cells plated at $3x10^5$ in 6 well plates were transfected with 2 µg of each p38 constructs (p38 wild type (WT), p38 dominant negative (DN), and p38 catalytically active (CA) (kindly provided by Dr. Douglas Gray, Ottawa Hospital Research Institute, Ottawa, Canada) using FugGENE HD Transfection Reagent (Roche, Mississauga, ON) as per manufacturer's protocol. Following 24 h, media was removed and replaced with media containing cisplatin (10µg/ml) treatment alone or in combination with SB203580 (10 µM) and cells were incubated for an additional 24 h. Cells were then harvested and analyzed by western blotting.

Adenovirus Infection. PC3 cells were plated at $2.5 X 10^5$ cells /well (6-well dish) and infected with $p53^{wt}$ or LacZ control adenovirus (provided by Dr. B. McKay, Ottawa Hospital Research Institute, Ottawa, Canada) at 25 plaque-forming units per millilitre (pfu)/cell. Following a 6 h infection period, media was removed from the cells and replaced with media containing cisplatin (10µg/ml) or taxol (25µM) for 24 h. Cells were then harvested and analyzed by Western blotting as described below.

Design and expression of small hairpin RNAs. The two 19mer sequences targeting ATF3 mRNA are; #1-5'-GCCAAAGAATATTCCATTT-3' and #2- 5'-GGGAGGGCCTGCAGTGATT-3' to pSuper vector from Oligoengine small hairpin RNA (shRNA) (#1: nucleotides 1524-1542; GenBank accession number NM_001030287. #2: nucleotides 1270-1289; GenBank accession number NM_001030287) target sequence. As controls, we used the GFP-targeted oligonucleotide 5'CATGCGTCCACTCTTCCTC-3' with accession number NC_011521. These sequences were BLAST confirmed for specificity. The forward and reverse synthetic 60 nt oligonucleotides (Integrated DNA Technologies, Coralville, IA) were designed, annealed, and inserted into the *Bgl*II/*Hind*III sites of pSUPER.retro.puro vector, following the manufacturer's instructions (Oligoengine, Seattle, WA). These constructs express a 19mer targeting two independent location within *ATF3* mRNA or GFP (control shRNA) mRNAs. Retroviral packaging cell line, RetroPack PT67 (Clonetech Labarotories, Mountain View, CA) was used for stable virus production according to the manufacturer's instructions. Briefly, packaging cells were transfected with ATF3-shRNA plasmids#1, #2 or GFP-shRNA, using FuGENE® HD Transfection Reagent (Roche, Mississauga, ON). After generation of stable clones and determination of viral titer, A549 cells were infected with viral supernatant using 4 μg/ml polybrene. Stable transfected clones expressing shRNAs were selected using 3μg/ml puromycin.

Western Blot Analysis. Cells plated at 0.7 X 10^6 / 60 mm dish were allowed to grow overnight and treated with indicated drug for 24 h. Protein samples were collected in RIPA buffer (50 mM Tris-CL pH 7.5, 150 mM sodium chloride, 1mM EDTA, 1% Triton-X-100, 0.25 % sodium deoxycholate, 0.1% SDS) containing 50 mM sodium fluoride, 1mM sodium orthovanadate, 10 mM β-glycerolphosphate and 1X Protease Inhibitor Cocktail (Sigma-

Aldrich, St. Louis, MO). Protein concentrations were assayed using Bio-Rad Protein Assay (Mississauga, Ontario, Canada) and a Biomate 3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Protein extracts representing 60 μg were separated on a 12% SDS-PAGE gel and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA). Membranes were blocked in 5% skim milk powder in Tris-buffered saline containing 10% Tween-20 (TBS-T) for 1h at room temperature followed by incubation with primary antibody diluted in 5% skim milk in TBS-T with shaking overnight at 4°C. Polyclonal antibody ATF3, ERK and phospho-ERK (Tyr204) were purchased from Santa Cruz, Santa Cruz, CA. Monoclonal anti-actin was purchased from Sigma-Aldrich, St. Louis, MO, and monoclonal anti-p53 (Ab-6) from Calbiochem, San Diego, CA. Polyclonal antibodies Jun, phospho-Jun (Ser73), p38, phospho-p38 (Thr180/Tyr182) and Parp were purchased from Cell Signalling Technology, Beverly, MA. Polyclonal antibodies against hsp27 and phospho-hsp27 (Ser78) were purchased from Stessgen, Ann Arbor, MI. Following washes in TBS-T, blots were incubated with the appropriate HRP-labelled secondary antibody for 1 hr at room temperature. Visualization of protein bands was performed using the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) exposed on Kodak film in a Konica Minolta SRX-101A tabletop processor.

RNA isolation and RT-PCR. MCF-7 cells plated at 0.8 X 10^6 cells per 10 cm dish were incubated at 37°C overnight. The next day cells were treated with cisplatin, in the absence or presence of the three MAPKinase inhibitors used in this study, for 24 h. Total RNA was extracted from cell samples using the RNAeasy1 kit (Qiagen, MD). RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). One

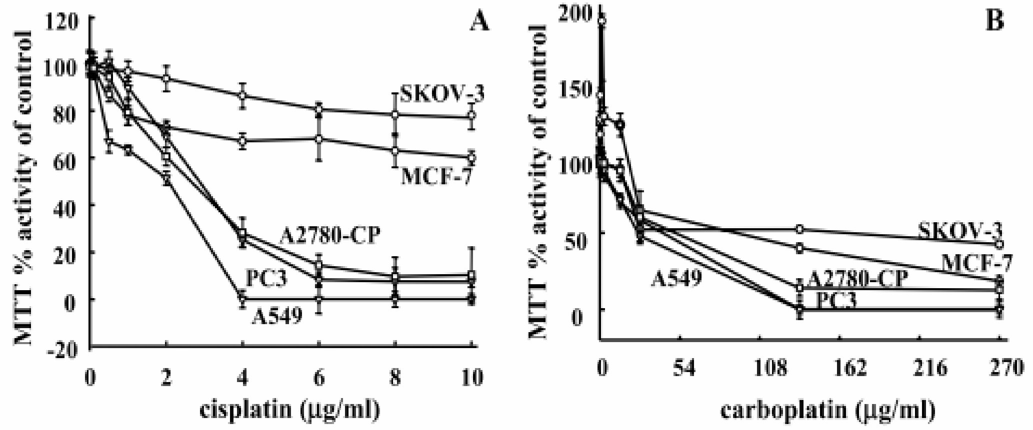
microgram of total RNA was reverse-transcribed to complementary DNA for quantitative, real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described [30.2]. The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City,CA) was used to detect amplification. A real-time PCR reaction was carried out in a total volume of 25 µl that contained 2.5 µl of synthesized cDNA (42 ng), 1.25 µl of Taq Man Gene Expression Assay Primer/Probe (20X) (Applied Biosystems, ATF3, HS00231069), 12.5 µl of Taq Man 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). Amplification conditions were 95°C for 5 min, 40 PCR cycles at 95°C for 15 sec and 60°C for 1 min. Three independent experiments were performed to determine the average gene expression and standard deviation.

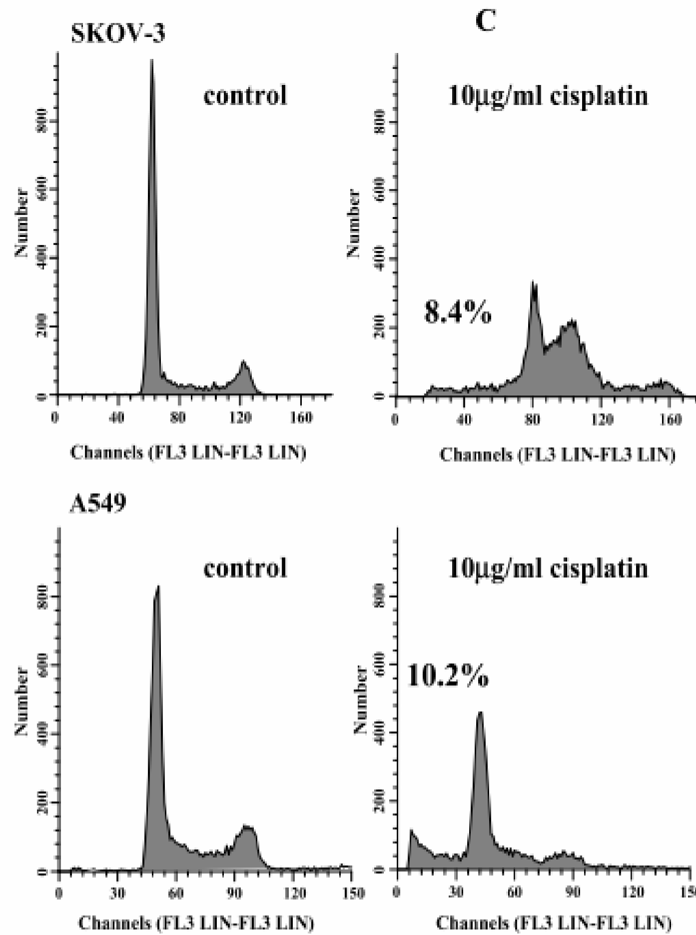
Results

Cisplatin and carboplatin cytotoxicity is associated with ATF3 induction

We analyzed the cytotoxic effects of cisplatin and carboplatin treatment on a number of human tumour cell lines, MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), SKOV-3 (ovarian carcinoma), PC3 (prostate carcinoma) and A2780-cp (ovarian carcinoma), using the MTT cell viability assay and flow cytometry (Figure 1.2). In A549, PC3 and A2780-cp cell lines higher doses of cisplatin lead to 100 % cytotoxicity (Figure 1.2A) whereas the MCF-7 and SKOV-3 cell lines appeared more resistant as determined by the MTT cell viability assay. Resistance to the cytotoxic effects of cisplatin in the MCF-7 and SKOV-3 cell lines was evidenced by a plateau patterning at higher doses of the treatment, a pattern previously suggested to be related to deficiency in pro-apoptotic factors [2.2] (Figure 1.2A). Indeed, the MCF-7 cell line is caspase-3 deficient [31.2]. Therefore, the observed resistance to cisplatin in the MCF-7 cell line may be mediated by a blockage in downstream apoptotic pathways in which the platinum–based drug acts. Carboplatin also induced cell cytotoxicity in all cell lines where MCF-7, SKOV-3, and A2780-CP cell line displayed the greatest resistance to the cytotoxic effects (Figure 1.2B). Interestingly, SKOV-3 and A2780-CP cell lines are functionally null for the tumour suppressor p53 which may contribute to their resistance. As expected, carboplatin was less cytotoxic at the same concentrations compared with cisplatin treatment. We further employed flow cytometry analyses to determine the potential for apoptosis induction by the platinum-based chemotherapeutics in the A549 and SKOV-3 cell lines. Apoptosis was visualized as a sub-2N peak that identifies apoptotic bodies resulting from cellular fragmentation [32.2, 33.2]. Cisplatin treatment (10μg/ml, 48 h) resulted in

Figure 1.2 Cisplatin and carboplatin treatment induce cytotoxicity and apoptosis in a panel of human cancer cell lines. Cell lines SKOV-3, MCF-7, A2780-cp, PC3, and A549 were treated with cisplatin (A) or carboplatin (B) for 48 hours, and cell viability was assessed as measured by MTT activity. Data are represented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars represent the standard deviation from six individual treated samples. (C) Flow cytometry analysis of SKOV-3 and A549 cell lines treated with cisplatin for 48 hours.

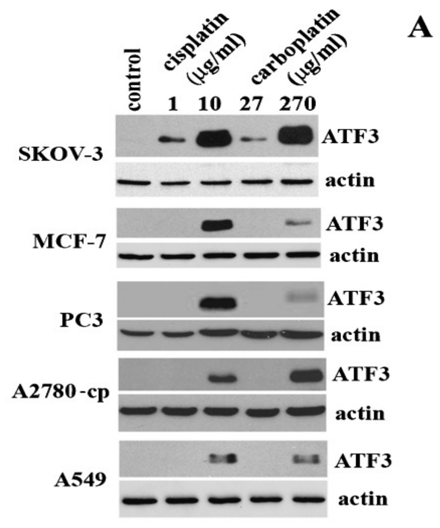

A
MTT % activity of control
120
100
80
60
40
20
0
-20
0
2
4
6
8
10
cisplatin (μg/ml)
SKOV-3
MCF-7
A2780-CP
PC3
A549
B
200
150
100
50
0
54
108
162
216
270
carboplatin (μg/ml)
SKOV-3
MCF-7
A549
A2780-CP
PC3

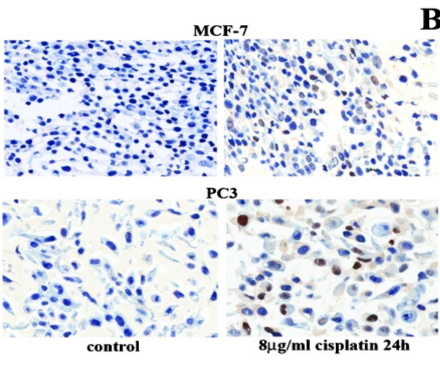

C
SKOV-3
control
10μg/ml cisplatin
8.4%
Number
Channels (FL3 LIN-FL3 LIN)
A549
control
10μg/ml cisplatin
10.2%
Number
Channels (FL3 LIN-FL3 LIN)

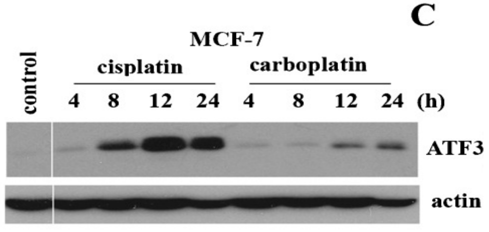
10.19 % and 8.42% of cells in the sub-2N fraction in A549 and SKOV-3 cell lines, respectively (Figure 1.2C). Differences between percentage of apoptotic cells observed between MTT assay and flow cytotometry assay are largely due to the qualitative nature for the flow cytotometry method [33.2]. Carboplatin treatment (270μg/ml, 48 h) resulted in 23.44 % and 11.33% of cells in sub-2N in A549 and SKOV-3 cell lines, respectively (data not shown).

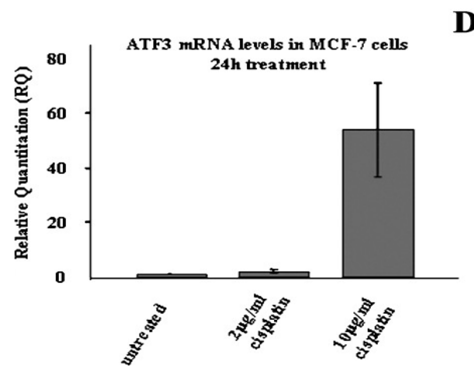
Previously our lab had identified lovastatin, a potent inhibitor of mevalonate synthesis, as an inducer of the ISR pathway and subsequent mediator of lovastatin-induced apoptosis [34.2]. Downstream effectors of the ISR pathway activated by lovastatin included members of the Activating Transcription Factor (ATF) family, ATF4 and ATF3. A role for the stress inducible gene, ATF3, in tumourgenesis has been demonstrated and can act as either a tumour suppressor or oncogene depending on cell context. Since various stress pathways that induce ATF3 expression have also been shown to regulate cytotoxicity, we first evaluated the potential of cisplatin and carboplatin to affect ATF3 expression. Indeed we found that ATF3 was significantly induced at the protein level when treated for 24 h with cytotoxic concentrations of cisplatin (10μg/ml) and carboplatin (270μg/ml) in a panel of human cancer cell lines (Figure 2.2A). These higher concentrations of drug treatment induced significant cytotoxicity at 48 h treatments as demonstrated by MTT assay analysis in all cell lines evaluated (Figure 1.2A). The non-cytotoxic doses of each drug tested (cisplatin, 1μg/ml; carboplatin, 2.7μg/ml) produced either a weak or undetectable induction of ATF3 (Figure 2.2A). Immunocytochemical analysis of ATF3 expression in cisplatin (8μg/ml) treated MCF-7 and PC3 cells for 24 h showed that while untreated cells did not express ATF3, a significant proportion of cisplatin treated cells showed expression and nuclear

Figure 2.2 ATF3 is induced by cisplatin and carboplatin. (A) ATF3 protein expression levels after treatment with low and cytotoxic doses of cisplatin (1 and 10 µg/ml) and carboplatin (27 and 270 µg/ml) in SKOV-3, MCF-7, PC3, A2780-cp, and A549 cell lines. (B) Immunocytochemistryanalysis of ATF3 expression (brown) in MCF-7 and PC3 cell lines in nontreated cells (control) and cisplatin treatment for 24 hours. Methylene blue stain is used as a nuclear counterstain. (C) Time course analysis of ATF3 expression in MCF-7 cells treated with cisplatin (10 µg/ml) or carboplatin (270 µg/ml) at the 4-, 8-, 12, and 24-hour time points. (D) ATF3 mRNA quantified by RT-PCR in MCF-7 cells untreated, treated with 2 and 10 µg/ml cisplatin for 24 hours. Error bars are representative of the standard deviation of quantified mRNA from three independent experiments. In all blots, actin is used as a loading control.


A
control
cisplatin (μg/ml)
carboplatin (μg/ml)
1 10 27 270
SKOV-3
ATF3
actin
MCF-7
ATF3
actin
PC3
ATF3
actin
A2780-cp
ATF3
actin
A549
ATF3
actin


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


C
MCF-7
control
cisplatin
carboplatin
4 8 12 24 4 8 12 24 (h)
ATF3
actin

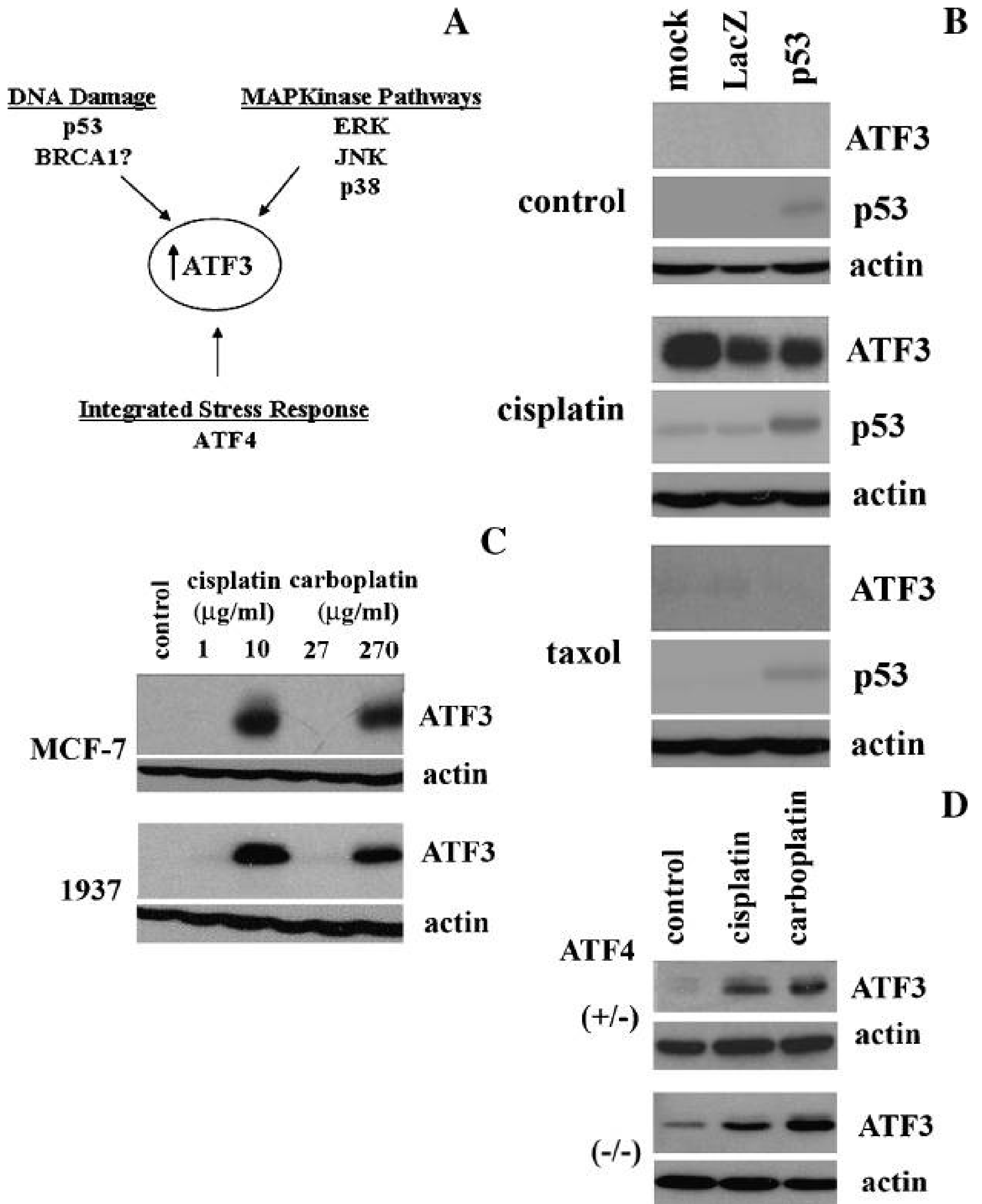

D
ATF3 mRNA levels in MCF-7 cells
24h treatment
Relative Quantitation (RQ)
0
20
40
60
80
untreated
2μg/ml cisplatin
10μg/ml cisplatin

localization of this transcription factor (Figure 2.2B). Time course analysis of ATF3 induction by cisplatin (10µg/ml) and carboplatin (270µg/ml) revealed maximal induction levels occurring at 12 and 24 h in the MCF-7 cell line (Figure 2.2C). Furthermore it was demonstrated that this cytotoxic cisplatin dose could specifically induce the levels of ATF3 mRNA (Figure 2.2D). In summary, ATF3 is highly induced at the protein and mRNA level by cytotoxic doses of these agents in human cancer derived cell lines.

Induction of ATF3 by cisplatin is independent of a p53, BRCA1, or ISR mechanisms

ATF3 mRNA and protein levels are readily induced by a wide range of stress causing agents [9.2]. The mechanism(s) of stress induced ATF3 has been previously well documented (Figure 3.2A) and in this study, we evaluated these regulatory mechanisms with respect to cisplatin induction of ATF3. Since the tumour suppressor p53 had been previously implicated in ATF3 regulation [22.2, 23.2] we determined its role in cisplatin induction of ATF3. ATF3 was induced by cisplatin in the p53 functionally null cell lines SKOV-3 and PC3, which suggested a p53-independent mechanism (Figure 2.2A). To further investigate a possible regulatory role, we virally expressed p53 in the PC3 cell line, treated the cells with cisplatin or taxol, a microtubule depolarizing agent, and determined ATF3 expression levels. ATF3 induction by cisplatin (10µg/ml, 24 h) was unchanged between LacZ vector control and p53 containing viral infection under cisplatin treatment further confirming a p53-independent mechanism (Figure 3.2B, middle panel). Taxol treatment (25µM, 24 h) had no effect on ATF3 expression levels (Figure 3.2B, bottom panel). We next looked at the possible involvement of the DNA damage response factor, BRCA1, in the regulation of ATF3 induction by platinum-based chemodrugs since previous reports suggested that

Figure 3.2 ATF3 induction by cisplatin is independent of a p53, BRCA1, and ISR mechanism. (A) Schematic representation of the potential mechanisms involved in ATF3 induction by cisplatin. (B) ATF3 detection in PC3 cells transduced with no virus (mock), LacZ vector control (LacZ), or p53wt (p53) containing adenovirus for 6 hours after no treatment (control; top panel) or after treatment with cisplatin (10 μg/ml; middle panel) or taxol (25 μM; bottom panel) for 24 hours. (C) ATF3 expression detected in MCF-7 and 1937 (BRCA1 null) cells untreated (control) or treated with cisplatin (1 and 10 μg/ml) or carboplatin (27 and 270 μg/ml) for 24 hours. (D) ATF3 detection in ATF4−/−and ATF4+/− MEFs untreated (control) or treated with cisplatin (1 μg/ml) and carboplatin (27 μM) for 24 hours. In all blots, actin is used as a loading control.


A
DNA Damage
p53
BRCA1?
MAPKinase Pathways
ERK
JNK
p38
ATF3
Integrated Stress Response
ATF4
B
mock
LacZ
p53
control
ATF3
p53
actin
cisplatin
ATF3
p53
actin
taxol
ATF3
p53
actin
C
control
cisplatin (μg/ml)
carboplatin (μg/ml)
1
10
27
270
MCF-7
ATF3
actin
1937
ATF3
actin
D
control
cisplatin
carboplatin
ATF4
(+/-)
ATF3
actin
(-/-)
ATF3
actin

BRCA1 could transcriptionally regulate ATF3 expression [17.2, 24.2]. In order to determine whether BRCA1 played a role in ATF3 induction by cis-or carbo-platin, ATF3 induction was contrasted in the breast adenocarcinoma cell lines expressing and null for BRCA1, MCF-7 and 1937, respectively. As previously demonstrated [35.2], we also confirmed the differential expression of BRCA1 in these cell lines and also showed that 1937 cells are sensitive to cisplatin and carboplatin at the higher cytotoxic doses employed (data not shown). As shown in Figure 3.2C, no difference in ATF3 induction levels was observed between the two cell lines with either treatment suggesting that induction of ATF3 by the chemotherapeutic drugs is independent of BRCA1 expression. Next we evaluated the role of the ISR pathway in mediating ATF3 induction since ATF3 is a downstream effector of the pathway [25.2]. We tested the ability of cisplatin and carboplatin to induce ATF3 expression in immortalized heterozygous or ATF4 null MEFs, the upstream inducer of ATF3 expression in the ISR pathway. Figure 3.2D demonstrates the absence of ATF4 had no effect on ATF3 induced by cis- and carbo-platin suggesting an ISR independent mechanism as well. Lower induction levels of ATF3 in the ATF4-deficient MEFs as compared with cell lines analyzed in Figure 2.2A, is likely due to lower doses of drug used (cisplatin;1µg/ml and carboplatin; 2.7µg/ml) due to enhanced sensitivity of these MEFs to the cytotoxic agents.

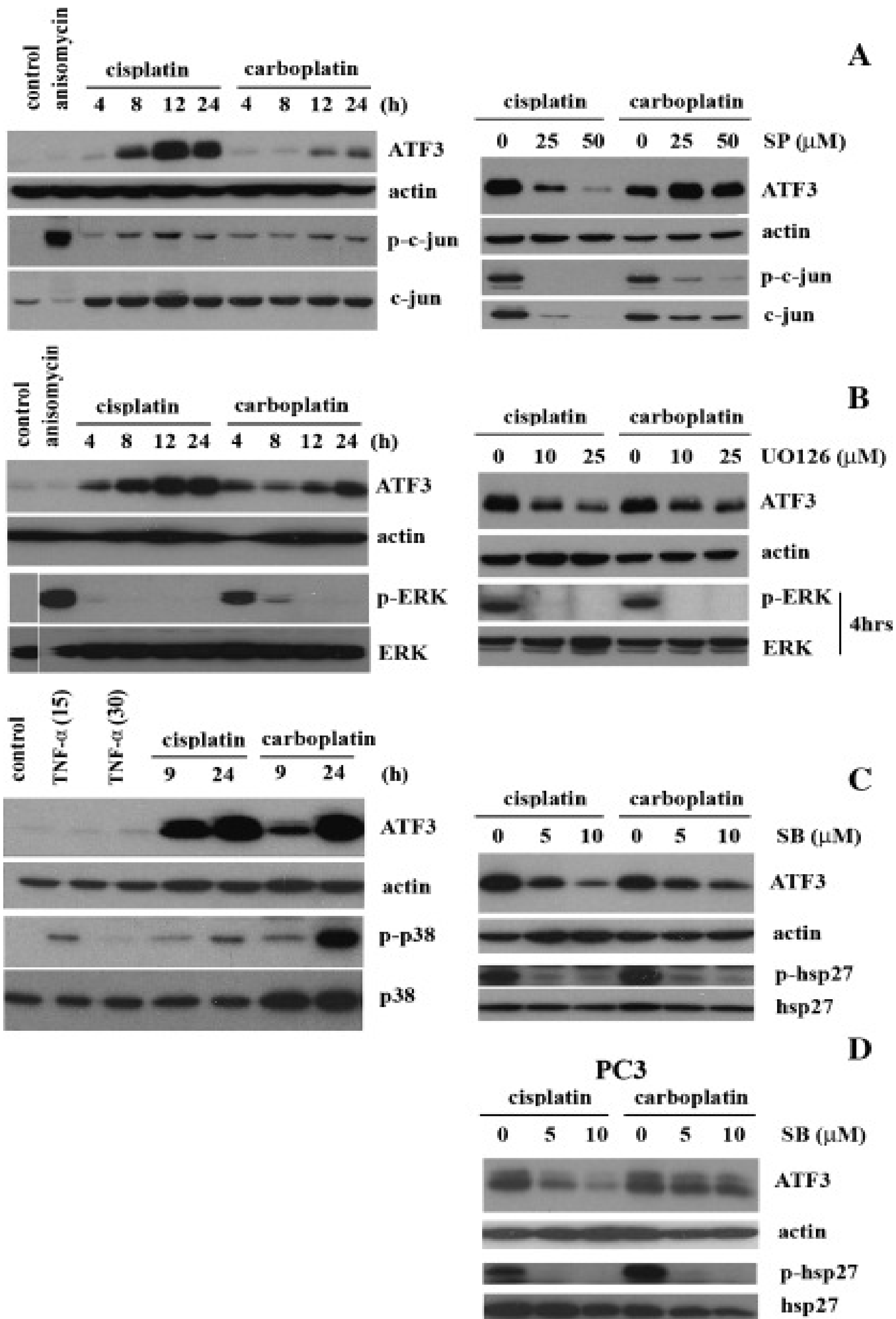
MAPKinase pathways regulate ATF3 induction by cisplatin.

Recent characterization of ATF3 induction by anisomycin, an antibiotic that activates multiple signalling pathways, revealed a MAPKinase dependent mechanism [27.2], therefore, we investigated the individual MAPKinase pathways for potential regulation of ATF3 expression by platinum-based chemotheraupeutic drugs. First we determined whether

the pathways were activated under the drug treatments in the MCF-7 cell line. Time course analysis of JNK pathway activation following treatment with platinum-based chemotherapeutic drugs as measured by the phosphorylation status of c-Jun, a downstream effector of the JNK pathway revealed a slight increase in phospho-c-Jun at 12 and 24 h which coincided with maximal ATF3 induction by both cisplatin and carboplatin (Figure 4.2A, left panel). To determine the role of the JNK pathway in ATF3 induction by cisplatin (10µg/ml) and carboplatin (270µg/ml), MCF-7 cells were treated with cis- or carbo-platin for 24 h in the presence of a JNK specific inhibitor, JNK Inhibitor II SP600125 (SP), which inhibited this pathway as measured by phospho-c-Jun levels (Figure 4.2A, right panel). ATF3 induction levels were found to be reduced under cisplatin treatment in the presence of JNK inhibitor, but not with carboplatin, in MCF-7 cells (Figure 4.2A, right panel).

Next we evaluated the role of the ERK pathway in ATF3 induction by cis- and carbo-platin. Time course treatment with chemodrugs revealed activation of the ERK pathway, as measured by the phosphorylation status of ERK, at 4h under cisplatin, and 4 and 8h under carboplatin treatment (Figure 4.2B, left panel). Phosphorylated ERK was not detected at 12 and 24 hrs under either treatment (Figure 4.2A, left panel). To determine the role of the ERK pathway in ATF3 induction by cisplatin and carboplatin, MCF-7 cells were treated with cis- or carbo-platin in the presence of the specific inhibitor to the ERK pathway, UO126, which was effective in blocking of the pathway as measured by phospho-ERK levels (Figure 4.2B, right panel). Treatment with ERK inhibitor in the presence of cis- and carbo-platin revealed a dose dependent decrease in ATF3 expression levels suggesting a role for the pathway in mediating induction by the chemodrugs (Figure 4.2B, right panel).

Figure 4.2 ATF3 induction by cisplatin is mediated by MAPK pathways in MCF-7 cells. (A) MCF-7 cells without treatment (control) and those treated with anisomycin (20 μM) for 1 hour and cisplatin (10 μg/ml) or carboplatin (270 μg/ml) for the 4-, 8-, 12-, and 24-hour time points were analyzed by Western blot analysis for the detection of ATF3, actin, phospho-c-jun (p-c-jun), and total c-jun (left panel). MCF-7 cells similarly treated with cisplatin or carboplatin in the absence (0) or presence of JNK pathway inhibitor (SP; 25 and 50 μM) for 24 hours were analyzed for the detection of ATF3, actin, p-c-jun, and total c-jun (right panel). (B) MCF-7 cells without treatment (control) and those treated with anisomycin (20 μM) for 1 hour and cisplatin or carboplatin as previouslymentioned for the 4-, 8-, 12-, and 24-hour time points were analyzed by Western blot analysis for the detection of ATF3, actin, phospho-ERK (p-ERK), and total ERK (left panel). MCF-7 cells treated with cisplatin or carboplatin in the absence (0) or presence of ERK pathway inhibitor (UO126; 10 and 25 μM) for 24 hours were analyzed for the detection of ATF3 and actin and at 4 hours for p-ERK and total ERK (right panel). (C) MCF-7 cells untreated (control), treated with tumor necrosis factor α (20 ng/ml) for 15 and 30 minutes, and cisplatin or carboplatin for the 9- and 24-hour time points were analyzed by Western blot analysis for the detection of ATF3, actin, phospho-p38 (p-p38), and total p38 (left panel). MCF-7 cells treated with cisplatin or carboplatin in the absence (0) or presence of p38 inhibitor, SB203580 (SB; 5 and 10 μM), for 24 hours were analyzed for the detection of ATF3, actin, phospho-hsp27 (p-hsp27), and total hsp27 (right panel). (D) PC3 cells treated with cisplatin (10 μg/ml) or carboplatin (270 μg/ml) in the absence (0) or presence of p38 inhibitor, SB203580 (SB; 5 and 10 μM), for 24 hours were analyzed for the detection of ATF3, actin, phospho-hsp27 (p-hsp27), and total hsp27.

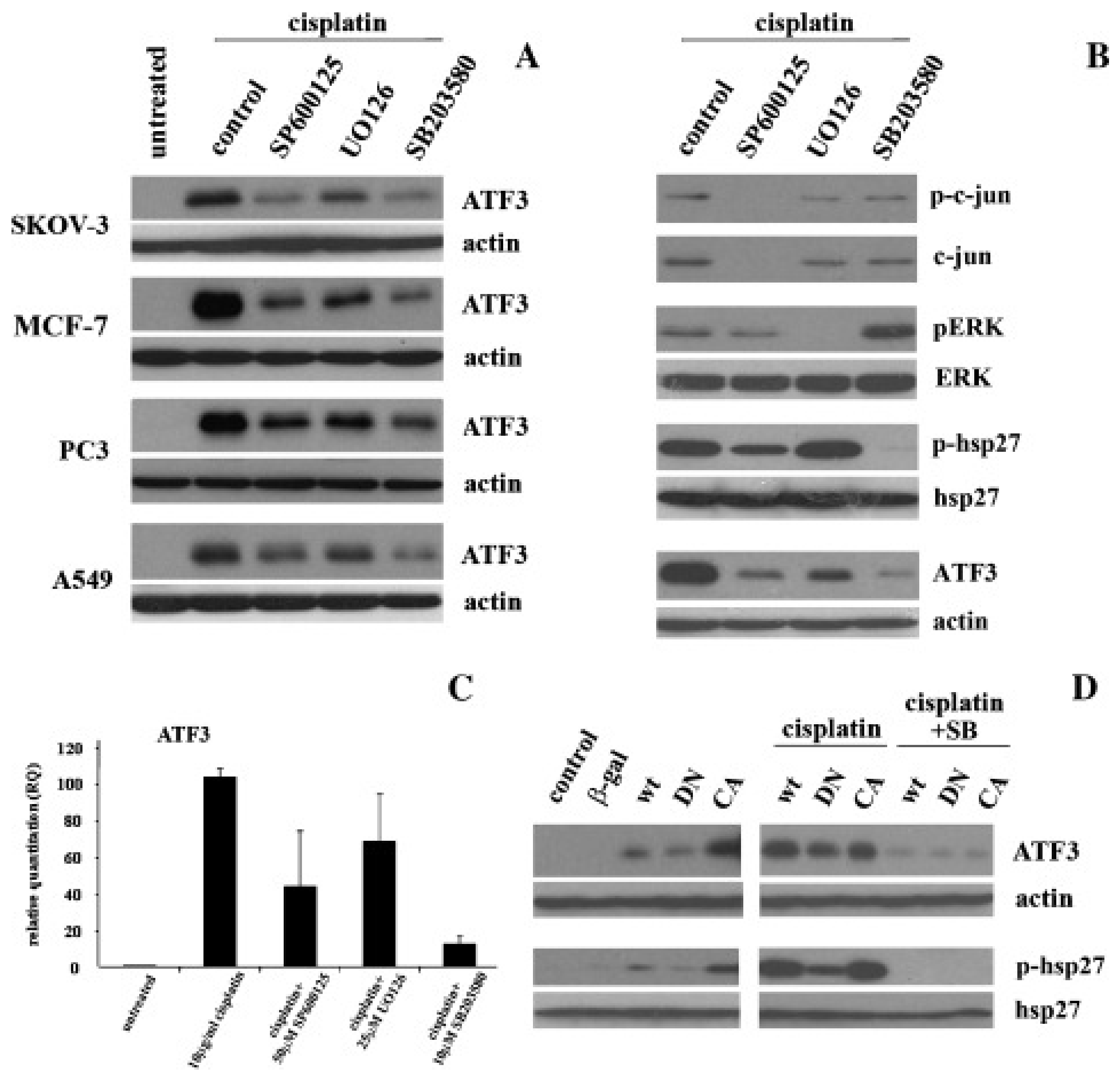


A
control
anisomycin
cisplatin
carboplatin
4 8 12 24 4 8 12 24 (h)
ATF3
actin
p-c-jun
c-jun
cisplatin
carboplatin
0 25 50 0 25 50 SP (μM)
ATF3
actin
p-c-jun
c-jun
B
control
anisomycin
cisplatin
carboplatin
4 8 12 24 4 8 12 24 (h)
ATF3
actin
p-ERK
ERK
cisplatin
carboplatin
0 10 25 0 10 25 UO126 (μM)
ATF3
actin
p-ERK
ERK
4hrs
C
control
TNF-α (15)
TNF-α (30)
cisplatin
carboplatin
9 24 9 24 (h)
ATF3
actin
p-p38
p38
cisplatin
carboplatin
0 5 10 0 5 10 SB (μM)
ATF3
actin
p-hsp27
hsp27
D
PC3
cisplatin
carboplatin
0 5 10 0 5 10 SB (μM)
ATF3
actin
p-hsp27
hsp27

Lastly, we determined the activation of the p38 pathway in MCF-7 cells following treatment with cis- or carbo-platin at 9 and 24 h time points. Indeed it was observed that the pathway was active at these time points as measured by the phosphorylation status of p38, which correlated with ATF3 induction levels (Figure 4.2C, left panel). To investigate the role of p38 pathway in the induction of ATF3 by cis- and carbo-platin MCF-7 cells were treated with chemodrugs in the presence of the specific p38 inhibitor, SB203580 (SB), for 24 h. This p38 inhibitor was shown to effectively block the pathway as measured by the phosphorylation status of the Heat Shock Protein 27 (hsp27), a downstream target of p38 (Figure 4.2C, right panel). ATF3 expression levels were also shown to be decreased by the inhibitor in a dose dependent manner suggesting a regulatory role of the p38 pathway in chemodrug induction of ATF3 (Figure 4.2C, right panel). Similarly, inhibition of the p38 pathway also resulted in effective blockage of the ATF3 induction by cis- and carbo- platin in the PC3 cell line (Figure 4.2D).

We similarly characterized the involvement of the MAPKinase pathways in cisplatin induced ATF3 expression in the tumour derived cells lines SKOV-3, MCF-7, PC3, and A459. These inhibitors against the three MAPKinase pathways evaluated had variable degrees of inhibition of ATF3 induction by cisplatin (10μg/ml, 24 h) implicating all three pathways in the mechanistic induction of ATF3 by cisplatin (Figure 5.2A). Interestingly, the pattern of reduced ATF3 induction by cisplatin in the presence of MAPKinase inhibitors was consistent between all four cancer cell lines with the p38 pathway inhibitor showing the greatest inhibition of ATF3 induction and the ERK pathway inhibitor showing the least (Figure 5.2A). To determine whether the MAPKinase pathway inhibitors used in this study were specific, cisplatin induced (10μg/ml, 24 h) activation of each pathway in the presence

Figure 5.2 Induction of ATF3 by cisplatin is mediated byMAPK pathways in a panel of human cancer cells. (A) SKOV-3, MCF-7, PC3, and A549 cells untreated and treated with cisplatin (10 μg/ml) for 24 hours in the absence (control) or presence ofMAPK pathway inhibitors (SP600125 [50 μM], UO126 [25 μM], and SB203580 (1)) and analyzed by Western blot analysis for ATF3 and actin. (B) MCF-7 cells treated with cisplatin (10 μg/ml) for 24 hours in the absence (control) or presence of MAPK pathway inhibitors (SP600125 [50 μM], UO126 [25 μM], and SB203580 (1)) and analyzed by Western blot analysis for p-c-jun, c-jun total, p-ERK, ERK total, p-hsp27, hsp27 total, ATF3, and actin. (C) ATF3 mRNA quantified by RT-PCR in MCF-7 cells untreated or treated with cisplatin (10 μg/ml) or cisplatin in the presence of SP (50 μM), UO126 (25 μM), and SB (10 μM) for 24 hours. Error bars represent the standard deviation of quantified mRNA from three independent experiments. Statistical analysis yielded p-values of > 0.05 for SP and UO126 treated cells, and > 0.001 for SB treated cells. (D) MCF-7 cells transiently transfected with 2 μg of p38 construct, p38-WT, p38-DN, and p38-CA, for 24 hours followed by no treatment or by treatment with cisplatin (10 μg/ml) alone or in combination with p38 inhibitor, SB (10 μM), for an additional 24 hours and analyzed by Western blot analysis for ATF3, actin, p-hsp27, and hsp27 total.



A
cisplatin
untreated
control
SP600125
UO126
SB203580
SKOV-3
MCF-7
PC3
A549
ATF3
actin
B
cisplatin
control
SP600125
UO126
SB203580
p-c-jun
c-jun
pERK
ERK
p-hsp27
hsp27
ATF3
actin
C
ATF3
relative quantitation (RQ)
120
100
80
60
40
20
0
D
control
β-gal
wt
DN
CA
cisplatin
cisplatin +SB
wt
DN
CA
wt
DN
CA
ATF3
actin
p-hsp27
hsp27

of these specific inhibitors was assessed. MCF-7 cells were treated with cisplatin alone, or in combination with JNK pathway inhibitor, SP600125 (50µM), ERK inhibitor, UO126 (25µM), or p38 pathway inhibitor, SB203580 (10µM), and activation of the pathways was assessed by phosphorylation status of c-jun (JNK pathway), ERK, and hsp27 (p38 pathway) (Figure 5.2B). Each pathway was inhibited by their respective inhibitor without significantly affecting the activation of the other pathways confirming their specificity (Figure 5.2B). We also demonstrate that the co-administration of all three MAPKinase inhibitors used in this study could down-regulate 10µg/ml cisplatin induced ATF3 expression at the mRNA level in a pattern of inhibition that was similar to that observed at the protein level at 24 h (Figure 5.2C).

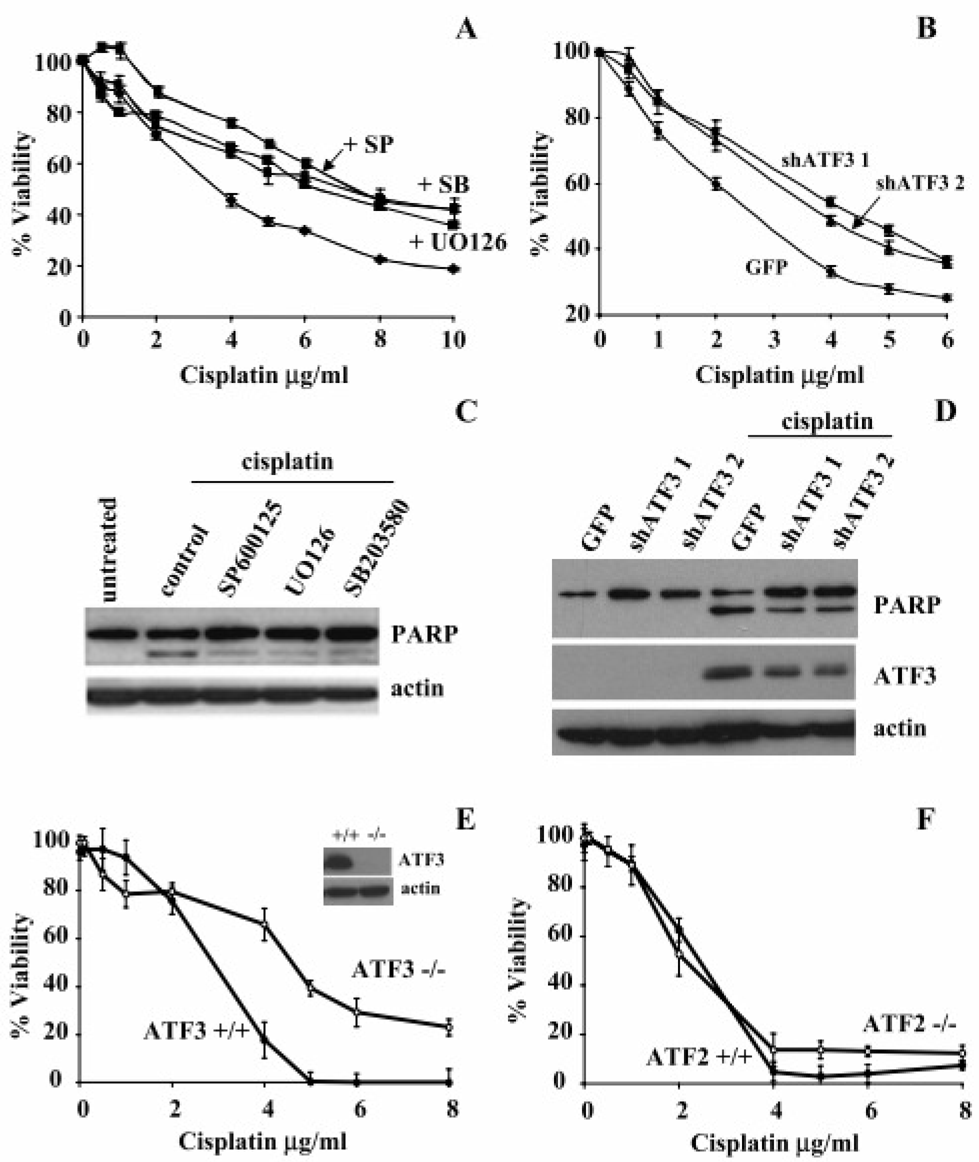
To further define the role of the p38 pathway in ATF3 induction by cisplatin, the MCF-7 cell line was transiently transfected with various p38 constructs (wild type; wt, dominant negative; DN and catalytically active; CA) for 24 h followed by no treatment (left hand panel) or 10µg/ml cisplatin in the absence or presence of 10µM SB203580 (right panel), for an additional 24 h. Untransfected (control) and a β-galactosidase expressing plasmid were used as negative controls (Figure 5.2D). In untreated samples, activation of the pathway as measured by phospho-hsp27 was highest in p38-CA transfected cells, and lowest in the p38-DN transfected cells, confirming functionality of the constructs. ATF3 expression levels were also increased upon p38-CA transfection, suggesting that activation of the p38 pathway is sufficient to induce ATF3 expression (Figure 5.2D, left panel). Following cisplatin treatment, phospho-hsp27 was induced, at greater levels, which is consistent with activation of the pathway by the drug (Figure 4.2C, left panel), where p38-DN transfected cells decreased expression of both p-hsp27 and ATF3 as compared with p38-WT and p38-

CA transfected cells (Figure 5.2D, right panel). Cisplatin treatment in the presence of the p38 inhibitor, resulted in abolishment of phospho-hsp27 and ATF3 expression irrespective of transfection status (Figure 5.2D, right panel). These data provide further evidence that ATF3 induction by cisplatin is mediated through MAPKinase pathway activation. Taken together these results identify the MAPKinase pathways as regulators of ATF3 induction by platinum-based cytotoxic drugs.

ATF3 regulates, in part, the cytotoxic effects of cisplatin

Since ATF3 has been previously shown to play a pro-apoptotic role in cancer models, we investigated the role of ATF3 induction by cisplatin in regulating the cytotoxic effects of cisplatin. Treating A549 cells with increasing concentrations of cisplatin in the presence or absence of SP600125 (50μM), U0126 (25μM), or SB203580 (10μM) the cytotoxic effects of cisplatin (48 h) was observed to be attenuated by approximately 20% as determined by the MTT cell viability assay (Figure 6.2A). Similar results were obtained in the PC3 cell line for the SB203580 inhibitor (data not shown). To further demonstrate ATF3's role as a factor in the cytotoxic affects of cisplatin, stable expression of shRNA against two different sequences of the ATF3-mRNA and to green fluorescent protein (GFP) as a negative control, were employed in the A549 cell line. Cells expressing both shATF3 and treated with cisplatin showed similar attenuation (20%) of the cytotoxic effects of the drug in the shATF3 cell lines as compared with shGFP control as determined by MTT analysis (Figure 6.2B). The cleavage status of PARP, a marker of apoptosis, was determined in the A549 cell line following treatment with cisplatin (10μg/ml, 24 h) in the presence or absence of the MAPKinase inhibitors at the above concentrations. Indeed, PARP cleavage induced by

Figure 6.2 ATF3 expression mediates, in part, the cytotoxic effects of cisplatin. (A) A549 cells treated with cisplatin (0-10 μg/ml) in the presence or absence of 50 μM SP600125, 25 μM UO126, and 10 μM SB203580 for 48 hours were assessed for cell cytotoxicity as measured by MTT activity. (B) A549 cells stably expressing shRNA against two separate ATF3 mRNA regions (shATF3-1 and shATF3-2) and GFP (negative control) were treated with cisplatin for 48 hours and were analyzed for MTT activity. For A and B, data are presented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars represent the standard deviation from six individual treated samples where all differences in cytotoxicity observed are statistically significant to p values of > 0.005. (C) A549 cells without treatment (untreated) or treated with 10 μg/ml cisplatin for 24 hours in the presence of MAPK inhibitors of 50 μMSP600125, 25 μM UO126, and 10 μM SB203580 and analyzed by Western blot analysis for PARP and actin as a loading control. (D) GFP, shATF3-1–, and shATF3-2–expressing cell lines treated with or without 10 μg/ml cisplatin for 24 hours and analyzed by Western blot analysis for PARP, ATF3, and actin expression. (E) ATF3−/−and ATF3+/+MEFs treated with cisplatin (0-8 μg/ml) and analyzed for MTT activity. All differences in cytotoxicity observed are statistically significant to p values of > 0.005. Western blot analysis for ATF3 and actin in ATF3−/− and ATF3+/+ MEFs treated with 10 μg/ml of cisplatin for 24 hours (inset). Actin was used as a loading control. (F) ATF2−/− and ATF2+/+ MEFs treated with cisplatin (0-8 μg/ml) and analyzed for MTT activity.


A
B
C
D
E
F
% Viability
Cisplatin μg/ml
+ SP
+ SB
+ UO126
shATF3 1
shATF3 2
GFP
cisplatin
untreated
control
SP600125
UO126
SB203580
PARP
actin
shATF3 1
shATF3 2
ATF3
+/+
-/-
ATF3 -/-
ATF3 +/+
ATF2 -/-
ATF2 +/+

cisplatin was reduced in the presence of MAPKinase inhibitors to all three pathways (Figure 6.2C) correlating with the attenuated cytotoxicity observed in Figure 6A. Likewise, reduced PARP cleavage was also observed in the shATF3 (shATF31 and shATF32) as compared with GFP control cell line (Figure 6.2D).

Lastly, we contrasted the cytotoxic effects of increasing cisplatin treatments on MEF cells expressing and knocked out for ATF3. ATF3 (+/+) MEFs were more sensitive to cytotoxic effects of cisplatin as compared with ATF3 (-/-) MEFs (Figure 6E). Cisplatin treatment induced ATF3 expression in the ATF3 (+/+) MEFs but not in the ATF3 (-/-) MEFs (Figure 6.2E, inset). The differences in cytotoxic effects of cisplatin observed in ATF3 (+/+) and (-/-) MEFs were contrasted in MEFs (+/+) and (-/-) for the ATF2 family member. No differences in the effect of cisplatin cytotoxicity was observed between ATF2 (+/+) and ATF2 (-/-) MEFs (Figure 6.2F). In all three models where ATF3 expression was targeted, there was approximately a 2 fold increase in cisplatin dose required for 50% cytotoxicity. Taken together, this study provides evidence that the cytotoxic effects invoked by cisplatin can in part be correlated to the drug's ability to induce ATF3 expression as regulated by MAPKinase pathways.

Discussion

In summary, this study has identified the platinum-based chemocytotoxic drug, cisplatin, as an inducer of the stress inducible gene, ATF3, at both the mRNA and protein level. Through elimination of potential ATF3 regulatory mechanisms namely, p53, BRCA1, and ISR, we identified ATF3 induction by cisplatin as regulated by the MAPKinase pathways JNK, ERK and p38. Inhibition of the MAPKinase pathway with the p38 inhibitor SB203580 in cisplatin treated cells resulted in the greatest decrease in ATF3 induction at the protein level in the human cancer cells analyzed. Increased ATF3 expression was associated with cisplatin-induced cytotoxicity as evidenced by attenuation of ATF3 expression and cytotoxicity with treatment of the three MAPK pathway inhibitors and in shATF3 knockdown cells compared with control. Targeting the activity of p38 with the expression of wild type, DN and CA expression constructs also modulated cisplatin induced ATF3 expression as the DN version attenuated cisplatin-induced ATF3 expression. Furthermore, ATF3 -/- MEFs were more resistant to cisplatin cytotoxicity as compared with ATF3 +/+ MEFs. Taken together these results provide strong evidence that ATF3 is a regulator of platinum-based chemotherapeutic induced cytotoxicity. Identifying ATF3 as a cisplatin induced gene through MAPKinase pathway activation may have therapeutic relevance. Inducers of the MAPKinase pathways or other cell stress pathways that enhance ATF3 expression may augment the cytotoxic effects of cisplatin. For example, two agents that induce ATF3 expression through different mechanisms, salubrinal and proteosome inhibitors, have demonstrated synergistic cytotoxicity in myeloma cells and may represent a novel combinational therapeutic approach [36.2, 37.2].

It is well characterized that the commencement of the anti-cancer effects of cisplatin involves DNA adduct formation resulting in apoptotic cell death if the DNA damage cannot

be adequately repaired [1.2]. However, the specific mechanism(s) downstream of cisplatin induced DNA damage which leads to the apoptotic response are poorly defined. In this study we show that cisplatin and its derivative, carboplatin, could readily induce ATF3 expression. A role for ATF3 in tumourigenesis has been implicated through its ability to affect the transcription of a number of regulators of apoptosis and cell proliferation including CHOP and cyclin D1, respectively [38.2, 39.2]. Depending on the cell type and the type and severity of the cell stressor, ATF3 has been implicated as both a proto-oncogene or tumour suppressor. For example, over-expression of ATF3 inhibited proliferation and induced cell cycle arrest in human cancer cells [17.2], whereas loss of ATF3 in a Ras transformed model resulted in higher proliferation rates and increased G1 to S phase transition efficiency [18.2]. Treating our panel of human cancer cell lines with a high and low dose of cisplatin or carboplatin revealed an increase in ATF3 protein expression which was associated with the high cytotoxic doses of these drugs implicating a role for ATF3 in regulating platin-induced cytotoxicity. Since ATF3 has been implicated as a biomarker for cell death in cancer models [16.2-19.2, 40.2], based on these results expression levels of ATF3 with respect to platin response in patients should be evaluated.

Following the identification of ATF3 as a cisplatin induced gene, the major objective of this study was to systematically identify the mechanism of induction of ATF3. A number of cellular stress pathways have been shown to regulate the expression of ATF3. The DNA damage response factors, p53 and BRCA1, had previously been linked to ATF3 regulation. Likewise, ATF3 is a known downstream effector of the ISR induced by ER, hypoxia, viral and metabolic stressors [25.2]. This study showed that neither BRCA1, p53, nor the ISR were factors in ATF3 induction by cisplatin. Instead the mechanism of induction of ATF3 by cisplatin was found to be largely MAPKinase pathway dependent. Looking at the

involvement of the ERK, JNK and p38 pathways we found all three pathways, when inhibited, lead to decreased induction of ATF3 by cisplatin. Although the inhibition of ATF3 induction by cisplatin in the presence of MAPKinase pathway inhibitors was significant, it was not complete suggesting that other regulatory mechanism(s) may exist.

In conclusion, we determined the functional relevance of ATF3 expression in regulating cisplatin induced cytotoxicity. Recent literature has implicated the MAPKinase pathways in the regulation of stress induced ATF3 apoptosis. Similar to our results, that ribotoxic stress induction of ATF3 was shown to be mediated through the p38 pathway and ATF3 expression was shown as a pro-apoptotic factor in HeLa cells [27.2]. Another recent study reported that cisplatin could induce ATF3 in T98G glioblastoma cells at both the protein and mRNA level [26.2]. In contrast to our results, this report showed that the induction of ATF3 by cisplatin was anti-apoptotic. Discrepancies between our results and others could stem from differences in cellular model or status of cell line malignancy both of which have previously been suggested to determine ATF3's role as an anti-or pro-apoptotic factor [18.2, 19.2].

The literature has previously reported that cisplatin treatment results in the activation of MAPKinases [41.2]. Reports showing activation of the p38 pathway by cisplatin has been exclusively correlated with pro-apoptotic outcomes in a number of cell lines, whereas activation of the JNK and ERK pathways are correlated with both anti- and pro- death outcomes [41.2]. Inhibition of the p38 pathway with specific inhibitors has been previously shown to increase resistance to cisplatin [42.2, 43.2]. Likewise, reduced activation of the p38 pathway has been identified as a mechanism correlated with cisplatin resistance [2.2]. Although the activation of MAPKinase pathways by cisplatin treatment has been documented the signalling pathways downstream of activation which determines cell fate are

poorly understood. This study identifies ATF3, previously defined as a factor capable of influencing cellular fate, as a novel target of the MAPKinase pathways when activated by cisplatin treatment. This study suggests ATF3 induction by cisplatin may identify a novel factor responsible for mediating the established link between cisplatin induced MAPKinase pathway activation and cell cytotoxic outcomes. Whether ATF3 is directly activated by the MAPKinase pathway or is induced downstream of known MAPKinase pathway transcription factor targets remains to be determined. Defining the specific mechanism(s) responsible for the anti-tumour affects of cisplatin may lead to novel and improved therapeutic approaches.

Footnote:

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Chapter 3: Results

Activating Transcription Factor 3 regulates in part the enhanced tumour cell cytotoxicity of the histone deacetylase inhibitor M344 and cisplatin in combination

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Abstract

Background

Activating Transcription Factor (ATF) 3 is a key regulator of the cellular integrated stress response whose expression has also been correlated with pro-apoptotic activities in tumour cell models. Combination treatments with chemotherapeutic drugs, such as cisplatin, and histone deacetylase (HDAC) inhibitors have been demonstrated to enhance tumour cell cytotoxicity. We recently demonstrated a role for ATF3 in regulating cisplatin-induced apoptosis and others have shown that HDAC inhibition can also induce cellular stress. In this study, we evaluated the role of ATF3 in regulating the co-operative cytotoxicity of cisplatin in combination with an HDAC inhibitor.

Results

The HDAC inhibitor M344 induced ATF3 expression at the protein and mRNA level in a panel of human derived cancer cell lines as determined by Western blot and quantitative RT-PCR analyses. Combination treatment with M344 and cisplatin lead to increased induction of ATF3 compared with cisplatin alone. Utilizing the MTT cell viability assay, M344 treatments also enhanced the cytotoxic effects of cisplatin in these cancer cell lines. The mechanism of ATF3 induction by M344 was found to be independent of MAPKinase pathways and dependent on ATF4, a known regulator of ATF3 expression. ATF4 heterozygote (+/-) and knock out (-/-) mouse embryonic fibroblast (MEF) as well as chromatin immunoprecipitation (ChIP) assays were utilized in determining the mechanistic induction of ATF3 by M344. We also demonstrated that ATF3 regulates the enhanced cytotoxicity of M344 in combination with cisplatin as evidenced by attenuation of cytotoxicity in shRNAs targeting ATF3 expressing cells.

Conclusion

This study identifies the pro-apoptotic factor, ATF3 as a novel target of M344, as well as a mediator of the co-operative effects of cisplatin and M344 induced tumour cell cytotoxicity.

Introduction

Alteration of gene expression plays a role in tumourigenesis and progression of cancer. Modulation of expression of genes such as tumour suppressors or oncogenes, are not exclusively due to mutations and can be manipulated through transcriptional regulation mechanisms which include DNA methylation and histone modification [1.3]. In cancer cells, the balance between histone acetylation and deacetylation catalyzed by histone acetyltransferases and histone deacetylases (HDAC), respectively, is often disrupted. For example, altered expression and aberrant recruitment of HDACs have been reported in tumours [2.3]. HDACs catalyze the removal of acetyl groups from histones resulting in chromatin condensation and transcriptional repression [1.3, 3.3, 4.3]. HDAC inhibitors act to reverse this transcriptional silencing of genes, which include tumour suppressors [1.3, 3.3, 4.3]. HDAC inhibitors are generally small molecule inhibitors that can readily diffuse across cellular membranes and directly interact with the zinc ion at the base of the catalytic pocket of this enzyme blocking substrate interaction and activity [1.3]. Coupled with their ability to induce cell cycle arrest, apoptosis, and disruption of angiogenesis, HDAC inhibitors have been evaluated as cancer therapeutic agents [1.3, 3.3, 4.3]. Currently the HDAC inhibitor, vorinostat, has been FDA approved for clinical use for treatment against cutaneous T-cell lymphoma [5.3].

cis-Diamminedichloroplatinum(II) (cisplatin) is among the most active anti-tumour agent used in human chemotherapy and widely used in various tumour types including lung and ovarian cancers [6.3]. Acquired resistance and toxicities associated with treatment are major impediments inhibiting their efficacy [7.3]. Cisplatin is primarily considered as a DNA-damaging agent that forms various types of bi-functional adducts in reaction with cellular DNA [6.3]. Cisplatin becomes activated intra-cellularly by the aquation of one of

two chloride leaving groups, and subsequently covalently binding to DNA, forming DNA adducts [8.3]. The final cellular outcome of DNA adduct formation is generally apoptotic cell death, thought to occur through halting of cellular processes such as replication and transcription leading to prolonged G2 phase cell-cycle arrest, and deregulation of signal transduction pathways involved in growth, differentiation, and stress responses [7.3]. Cellular mechanisms of resistance to platinum-based chemotherapeutics are multifactorial and contribute to severe limitation in their use in clinical practice. They include molecular events inhibiting drug-DNA interaction, such as a reduction in cisplatin accumulation inside cancer cells or inactivation by thiol-containing species [6.3]. Other mechanisms of resistance acting downstream to the initial reaction of cisplatin with DNA, include an increase in adduct repair and a decrease in induction of apoptosis [7.3].

Pre-clinical and clinical studies have demonstrated that HDAC inhibitors can enhance the anticancer activity of a variety of epigenetic as well as chemotherapeutic agents including cisplatin [2.3, 9.3]. For example, promising clinical trials combining platins as well as other chemotherapeutics with HDAC inhibitors have been conducted [10.3, 11.3]. The ability of HDAC inhibitors to enhance the anti-cancer activity of known chemotherapeutic drugs is believed to be related to their function as positive regulators of gene transcription. As such, HDAC inhibitors have pleiotropic effects and can alter the expression of a wide variety (1000s) of genes [1.3-3.3]. In particular, HDAC inhibitor treatment has been shown to augment expression of genes such as the cell cycle suppressor, p21, apoptotic factors related to both extrinsic (death receptors and ligands) and intrinsic (Bcl-2 family members) pathways, and angiogenic factors such as HIF1α and VEGF [1.3-3.3].

It is well established that HDAC inhibitors can enhance the anticancer activity of cisplatin in vitro in a variety of cancer cell models [12.3-17.3] . Few studies exist, however, detailing the mechanism of enhanced anti-cancer effects by HDAC inhibitors in combination with cisplatin. For example, Rikiishi et al, correlated enhanced cytotoxicity by HDAC inhibitors in combination with cisplatin with reduced levels of the antioxidant intracellular reduced glutathione (GSH) in an oral squamous cell carcinoma model [18.3]. Our recent work has demonstrated that cisplatin treatment induces Activation of Transcription Factor (ATF) 3, a member of the ATF/cyclic AMP response element-binding family to regulate cisplatin-induced cytotoxicity [19.3]. ATF3 expression is induced by a wide variety of stress causing agents including hypoxia, metabolic stress and DNA damage [20.3]. ATF3 is also induced in times of physiological stress such as liver regeneration [21.3], brain seizure [22.3], ischemia-reperfusion of the heart and kidney [23.3], and nerve damage [24.3]. ATF3 has been shown to play a role in apoptosis and proliferation, two cellular processes critical for cancer progression [25.3-28.3]. Divergence in function of ATF3 between a pro-and anti-apoptotic factor in cancer models is dependent on both cellular model and state of malignancy [8.3, 25.3, 28.3]. Activation of ATF3 by a wide array of stress signalling pathways have been demonstrated including DNA repair pathway components p53 [29.3, 30.3], the integrated stress response (ISR) that is principally activated by hypoxia and metabolic stress [31.3], and the MAPKinase cascades (SAPK/JNK, p38 and ERK) [32.3, 33.3].

In this study we identify a novel target of the HDAC inhibitor M344, showing that treatment induced up-regulation of the stress inducible transcription factor ATF3. We show that M344 treatment can induce ATF3 expression at the protein and mRNA level in a panel of human derived cell lines. We also show that combination treatment with cisplatin and

M344 could enhance induction of ATF3 compared with cisplatin alone. Likewise, M344 treatment increased the cytotoxic effects of cisplatin in the human cancer cell lines. Unlike cisplatin whose mechanistic induction of ATF3 was shown previously to be dependent on the MAPKinase pathways [19.3], ATF3 induction by M344 was found to be independent of the MAPKinase pathways and reliant on the ISR pathway. Finally, we correlated increased ATF3 expression with the enhanced cytotoxicity of M344 in combination with cisplatin utilizing ATF3 shRNA expressing cell lines. Taken together, this study identifies the pro-apoptotic factor, ATF3 as a novel target of HDAC inhibitors, as well as a novel factor regulating the co-operative effects of cisplatin and HDAC inhibitor induced cytotoxicity.

Material and Methods

Tissue Culture. The A549, PC3, and MCF-7 cell lines were obtained from American Type Culture Collection (ATCC; Rockville, MD). The SK-OV3 cell line was kindly provided by Dr. Barbara Vanderhyden, Ottawa Hospital Research Institute (OHRI), Ottawa, Canada. The MEFs used in this study were derived from heterozygote (+/-) and knockout mice (-/-) from an ATF4 murine model (kindly provided by D. Park, University of Ottawa, Ottawa, Ontario). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Medicorp, Montreal, Canada) and 100 units penicillin and 100μg streptomycin (GIBCO, Burlington, ON) / ml of media. ATF4 (-/-) MEFs were maintained in DMEM containing 10% fetal bovine serum, 0.1mM nonessential amino acids, 55μM 2-mercaptoethanol, and 100units penicillin and 100μg streptomycin/ml of media. Cells were exposed to the HDAC inhibitor, 4-(Dimethylamino)-N-[7-(hydroxyamino)-7-oxoheptyl]-benzamide (M344) (Sigma, St. Louis, MI), or cisplatin (provided by the pharmacy at the Ottawa Hospital Regional Cancer Centre, Ottawa) alone or in combination with the p38 inhibitor SB203580 (Calbiochem, Gibbstown, NJ), JNK inhibitor, JNK inhibitor II (SP600125) (Calbiochem) or ERK inhibitor UO126 (Calbiochem) diluted in DMSO.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay. In a 96-well flat-bottomed plate (Nunc, Naperville, IL) ~5,000 cells/150μL 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 indicated drugs and incubated for 48 hrs at 37°C. Following treatment, 42μL of a 5mg/mL solution in PBS of the MTT tetrazolium substrate (Sigma) was added to each well and incubated for ~ 20min at 37°C. The resulting violet formazan precipitate was solubilised by the addition of 82μL of a 0.01 mol/L HCl/10% SDS

(Sigma) solution, and allowed to further incubate at 37°C overnight. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies (West Sussex, UK) at 570 nm to determine the absorbance of the samples.

Design and expression of small hairpin RNAs. The two 19mer sequences targeting ATF3 mRNA are; #1-5'-GCCAAAGAATATTCCATTT-3' and #2- 5'-GGGAGGGCCTGCAGTGATT-3' to pSuper vector from Oligoengine small hairpin RNA (shRNA) (#1: nucleotides 1524-1542; GenBank accession number NM_001030287. #2: nucleotides 1270-1289; GenBank accession number NM_001030287) target sequence. As controls, we used the GFP-targeted oligonucleotide 5'CATGCGTCCACTCTTCCTC-3' with accession number NC_011521. These sequences were BLAST confirmed for specificity. The forward and reverse synthetic 60 nt oligonucleotides (Integrated DNA Technologies, Coralville, IA) were designed, annealed, and inserted into the *Bgl*II/*Hind*III sites of pSUPER.retro.puro vector, following the manufacturer's instructions (Oligoengine, Seattle, WA). These constructs express a 19mer targeting two independent location within *ATF3* mRNA or GFP (control shRNA) mRNAs. The retroviral packaging cell line, RetroPack PT67 (Clonetech Laboratories, Mountain View, CA) was used for stable virus production according to the manufacturer's instructions. Briefly, packaging cells were transfected with ATF3-shRNA plasmids#1, #2 or GFP-shRNA, using FuGENE® HD Transfection Reagent (Roche, Mississauga, ON). After generation of stable clones and determination of viral titre, A549 cells were infected with viral supernatant using 4µg/ml polybrene. Stable transfected clones expressing shRNAs were selected using 3µg/ml puromycin.

Western Blot Analysis. Cells plated at 0.7 X 10^6 per 60 mm dish were allowed to grow overnight and treated with indicated drug for 24 hrs. Protein samples were collected in RIPA buffer (50mM Tris-CL pH 7.5, 150mM sodium chloride, 1mM EDTA, 1% Triton-X-100, 0.25% sodium deoxycholate, 0.1% SDS) containing 50mM sodium fluoride, 1mM sodium orthovanadate, 10mM β-glycerolphosphate and 1X Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentrations were assayed using Bio-Rad Protein Assay (Mississauga, Ontario, Canada) and a Biomate 3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Protein extracts representing 40μg were separated on a 10% SDS-PAGE gel and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA). Membranes were blocked in 5% skim milk powder in Tris-buffered saline containing 10% Tween-20 (TBS-T) for 1 hr at room temperature followed by incubation with primary antibody diluted in 5% skim milk in TBS-T with shaking overnight at 4°C. Polyclonal antibody ATF3 was purchased from Santa Cruz, Santa Cruz, CA. Monoclonal anti-actin was purchased from Sigma-Aldrich, St. Louis, MO. Polyclonal antibody to PARP was purchased from Cell Signalling Technology, Beverly, MA. Polyclonal antibodies against HSP27 and phospho-HSP27 (Ser78) were purchased from Stessgen, Ann Arbor, MI. Following washes in TBS-T, blots were incubated with the appropriate HRP-labelled secondary antibody for 1 hr at room temperature. Visualization of protein bands was performed using the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) exposed on Kodak film in a Konica Minolta SRX-101A tabletop processor.

RT-RNA isolation and RT-PCR. MCF-7 cells plated at 0.8 X 10^6 cells per 10cm dish were incubated at 37°C overnight. The next day cells were treated with either with M344, cisplatin or their combination for 24 hrs. Total RNA was extracted using the RNeasy1 kit (Qiagen, Mississauga, ON). RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). One microgram of total RNA was reverse-transcribed to complementary DNA for quantitative, real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described [34.3]. The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification. Real-time PCR reactions were carried out in a total volume of 25µl that contained 2.5 µl of synthesized cDNA (42ng), 1.25 µl of TaqMan Gene Expression Assay Primer/Probe (20X) (Applied Biosystems, ATF3, HS00231069), 12.5µl of TaqMan Universal PCR Master Mix (2X) (Applied Biosystems, 4304437) and 8.75µl of RNase-free water for ATF3 expression. The endogenous control for ATF3 was the housekeeping gene, human GAPDH (20X) (Applied Biosystems, HS4333764-F). Amplification conditions were 95°C for 5 min, 40 PCR cycles at 95°C for 15sec and 60°C for 1min. Three independent experiments were performed to determine the average gene expression and standard deviation.

Chromatin Immunoprecipitation (ChIP) Assay

Cells treated for 24 hrs in 10cm dishes were fixed with 1% formaldehyde (BDH, VWR International, Mississauga, ON) for 20min at room temperature in order to cross-link the DNA and protein. The cross-linking was quenched by adding glycine to a final concentration of 200mM and incubating at room temperature for 5min. Cells were then washed twice with ice-cold PBS and harvested in 1mL cold PBS by centrifugation at 4ºC

for5 min at 5,000 rpm. The pellet was resuspended in 90µL lysis buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1% SDS) supplemented with 1x Protease Inhibitor Cocktail (Sigma-Aldrich), 1mM 1,4-dithio-DL-threitol (DTT) (Sigma-Aldrich), and 1mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The lysates were sonicated using a Sonicator 3000 (Misonix, Farmingdale, NY) at power setting #1 for a total of 3min on ice with 10 sec on/off pulses to shear the DNA to an average size of 300 to 1000 base pairs. Sonicated lysates were cleared of debris by centrifugation for 15min at 14,000rpm at 4ºC. 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 (20mM Tris-HCl pH 8.0, 150mM NaCl, 2mM EDTA pH 8.0, 1% Triton X-100) supplemented with 1x Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), and 1mM PMSF (Sigma-Aldrich). Positive sample cell lysates were immunoprecipitated by overnight rotation at 4ºC with rabbit anti-acetyl H4 (1:200, Millipore) 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 40µL of protein A agarose/salmon sperm DNA 50% slurry (Millipore) to both samples and negative controls. The agarose beads/immune complexes were then pelleted gently by centrifugation for 1 min at 3,000rpm at 4ºC. The beads were washed with 1mL of the following buffers by rotation for 10 min at 4ºC, then pelleted gently by centrifugation for 1 min at 3,000 rpm at 4ºC, discarding the supernatant following each wash: Buffer A (low salt; 0.1% SDS, 1% Triton X-100, 20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 150mM NaCl) once, Buffer B (high salt; 0.1% SDS, 1% Triton X-100, 20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 500mM NaCl) once, Buffer C (1% NP-40, 1% sodium deoxycholate, 20mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 0.25M LiCl) once, TE washing buffer (10mM Tris-

HCl pH 8.0, 1mM EDTA pH 8.0) twice. Freshly prepared elution buffer (1% SDS, 100mM $NaCHO_3$) was added to all samples (input and negative controls, and samples) to a final volume of 400µL and samples were rotated at room temperature for 30 min. The agarose beads were removed from the samples by centrifugation for 1 min at 3,000 rpm.

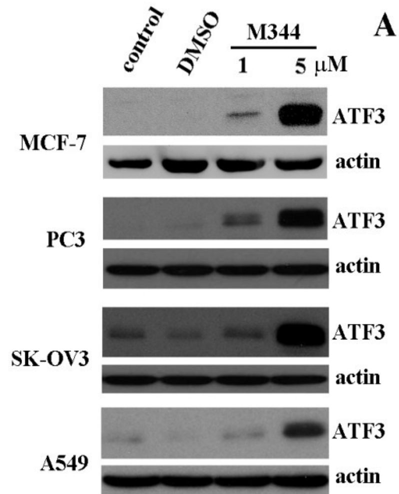
The DNA-protein cross-linking was reversed by overnight incubation with 5µL proteinase K (20mg/mL, Roche Diagnostics, Laval, QC, CAN) at 65ºC. The DNA was purified using a QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was eluted in 50µL ddH_2O and samples were stored at -80ºC. Conventional PCR was performed with amplification conditions as follows; 95°C for 2 min, 40 PCR cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and finally 72°C for 5 min. The binding of acetyl H4 to the ATF3 and p21 proximal promoter regions were determined using the following primer pairs: ATF3, forward-5' CCGAACTTGCATCACCAGTGC, reverse-5' GAGCTGTGCAGTGCGCGCC; p21, forward-GGTGTCTAGGTGCTCCAGGT, reverse –GCACTCTCCAGGAGGACACA [35.3]. PCR products were resolved on 1.6% agarose gels.

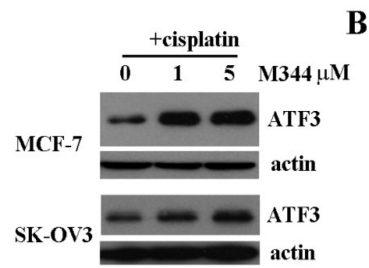
Results

HDAC inhibition induces ATF3 expression and enhances cisplatin cytotoxicity.

We have recently demonstrated that ATF3 expression plays a role in cisplatin induced cytotoxicity [19.3]. Given the emerging role of HDAC inhibitors as anti-cancer agents, we evaluated whether their activities also regulate ATF3 expression. Indeed we found that M344 treatment, a potent pan HDAC inhibitor, could affect ATF3 expression following 24hrs treatment. The higher (5μM) dose of M344 in a panel of human derived cancer cell lines, MCF-7 (breast adenocarcinoma), PC3 (prostate carcinoma), SK-OV3 (ovarian carcinoma), and A549 (lung carcinoma) demonstrated consistent up-regulation of ATF3 protein expression (Figure 1.3A). Since our previous work had shown that cisplatin could also induce ATF3 expression, we evaluated ATF3 expression following combinational treatment with M344 and cisplatin. M344 treatment (1 and 5μM) in combination with cisplatin (10μg/ml) for 24 hrs enhanced induction of ATF3 compared with cisplatin treatment alone as determined by Western blot analysis (Figure 1.3B). M344 induction of ATF3 expression was also evaluated at the mRNA level in the MCF-7 cell line and found to be similarly induced under these experimental conditions (Figure 1.3C). Differences in ATF3 mRNA expression, although not statistically significant likely due to high variability of transcript induction between experiments, was generally additive in combination treatments compared with M344 and cisplatin treatment alone (Figure 1.3C). Since it has been shown that HDAC inhibitors can enhance the cytotoxicity of cisplatin, we confirmed this previous observation in the MCF-7 and SK-OV3 cell lines where combination treatment lead to approximately 20% increased cytotoxicity compared with cisplatin treatment alone

Figure 1.3 M344 induces ATF3 and enhances its expression in combination with cisplatin treatment. A, ATF3 protein expression levels following 24 hr treatment with control, DMSO vehicle, and, low and high doses of M344 (1 and 5 μM) in MCF-7, PC3, SKOV-3 and A549 cell lines. B, ATF3 protein expression levels following 24 hrs treatment with cisplatin (10 μg/ml), or cisplatin in combination with M344 (1 and 5μM) in MCF-7 and SKOV-3 cell lines. In all blots actin is used as a loading control. C, ATF3 mRNA quantified by quantitative RT-PCR of MCF-7 cells comparing untreated treated control, DMSO vehicle, M344 (1 and 5μM), cisplatin (10μg/ml), or cisplatin in combination with M344 (5μM) for 24 hrs. Error bars represent the standard deviation of quantified mRNA from three independent experiments normalized to GAPDH expression.


control
DMSO
M344
1
5 μM
A
MCF-7
ATF3
actin
PC3
ATF3
actin
SK-OV3
ATF3
actin
A549
ATF3
actin


B
+cisplatin
0
1
5
M344 μM
MCF-7
ATF3
actin
SK-OV3
ATF3
actin

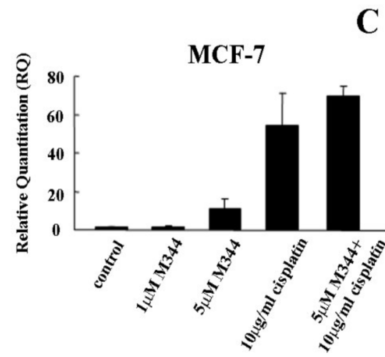
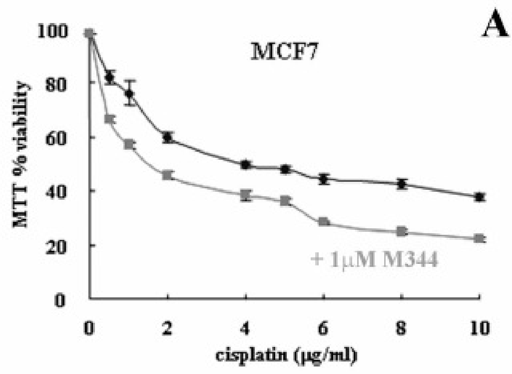
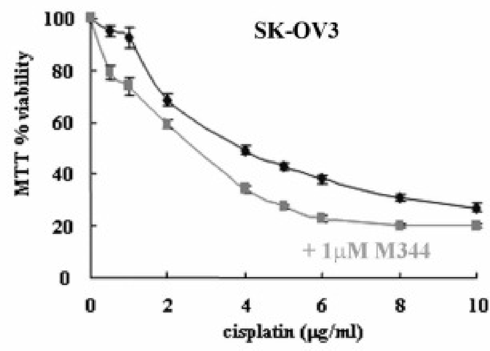
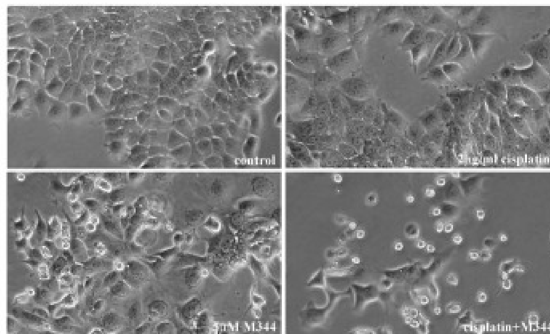

C
MCF-7
Relative Quantitation (RQ)
80
60
40
20
0
control
1μM M344
5μM M344
10μg/ml cisplatin
5μM M344+
10μg/ml cisplatin

Figure 2.3 M344 enhances the cytotoxicity of cisplatin. A, MCF-7 and SK-OV3 cell lines treated with cisplatin (0–10μg/ml) in the presence (grey) or absence (black) of M344 (1μM) for 48 hrs was assessed for cell viability employing the MTT assay. Data is represented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars represent the standard deviation from six individual treated samples from two independent experiments where all differences in cytotoxicity were found to be statistically significant to a p-value of > 0.005. B, Phase contrast images of MCF-7 cells treated with no treatment (control), cisplatin (2μg/ml), M344 (1μM) and cisplatin and M334 in combination for 48 hrs.


A
MCF7
MTT % viability
+ 1μM M344
cisplatin (μg/ml)


SK-OV3
MTT % viability
+ 1μM M344
cisplatin (μg/ml)

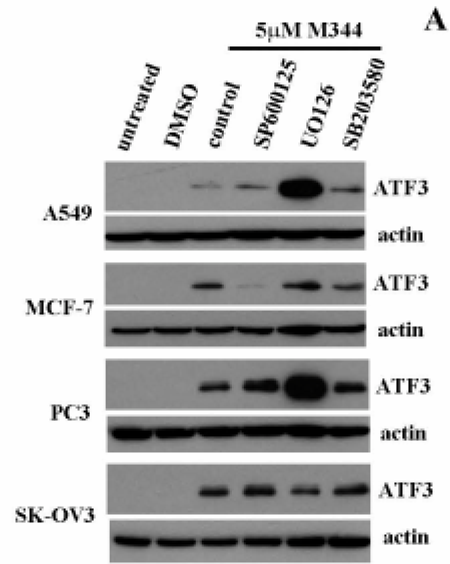

B
control
cisplatin+M344

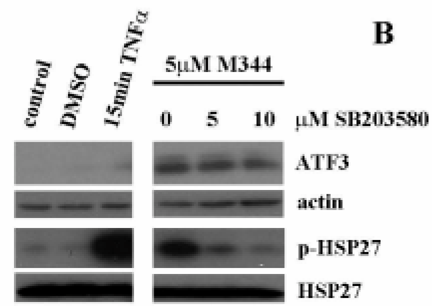
(Figure 2.3A) as measured by the MTT cell viability assay. The observed enhanced cytotoxicity was also demonstrated by cell imaging following either cisplatin, M344 alone, or in combinational treatment in the MCF-7 cell line for 48 hrs (Figure 2.3B). A low dose of cisplatin was used (2μg/ml) which does not induce significant cytotoxicity in the MCF-7 cell line (Figure 2.3A, top panel) however, following combination treatment with M344 (5μM) enhanced cytotoxicity was clearly evident in the corresponding phase contrast images (Figure 2.3B). In summary, these data demonstrate that M344 is a novel inducer of ATF3 and an enhancer of ATF3 induction when in combination with cisplatin treatment. Increased ATF3 expression mediated by combinational treatment correlates with increased cytotoxicity compared with cisplatin alone.

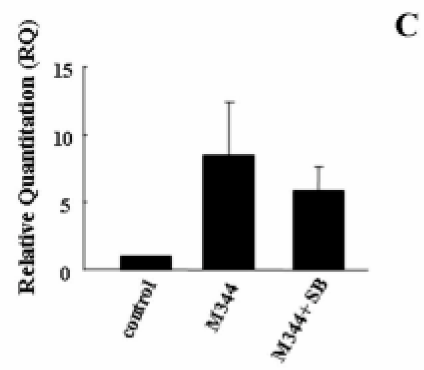
ATF3 induction by M344 is regulated by the Integrated Stress Response.

Next, we evaluated a number of cell signalling pathways that are known regulators of ATF3 expression to determine the mechanism of induction of ATF3 by M344. Our previous work had identified the MAPKinase pathways as mediators of ATF3 induction by cisplatin. Similarly, other groups had shown the involvement of MAPKinase pathways in mediating ATF3 induction through other stress inducing agents [32.3]. We evaluated the role of all the MAPKinase pathways using inhibitors to the JNK (SP600125), and ERK (UO126) as well as p38 (SB203580) pathways in all the cell lines used in this study. Unlike our previous data which showed that all inhibitors to these pathways could down regulate the induction of ATF3 by cisplatin consistently in all the same cell lines (MCF-7, PC3, SKOV-3 and A549), these inhibitors did not affect ATF3 induction by M344 treatment. This data essentially

Figure 3.3 ATF3 induction by M344 is independent of MAPKinase pathways. A, A549, MCF-7, PC3 and SKOV-3 cells untreated (control), and treated with DMSO, or M344 (5μM) for 24 hrs in the absence (control) or presence of MAPKinase pathway inhibitors SP600125 (50μM), UO126 (25μM), and SB203580 (10μM) and analyzed by Western blotting for ATF3 and actin. B, MCF-7 cells untreated (control), treated with DMSO, TNFα for 15min, or with M344 (5μM) in the absence or presence of p38 inhibitor, SB203580 (5 and 10μM) for 24 hrs were analyzed for ATF3, actin, phospho-HSP27 (p-hsp27), and total HSP27 expression by Western blotting. C. ATF3 mRNA quantified by quantitative RT-PCR of MCF-7 cells untreated (control), treated with M344 (5μM) or M344 in the presence of SB203580 (10μM) for 24 hrs. Error bars represent the standard deviation of quantified mRNA from three independent experiments.


A
5μM M344
untreated
DMSO
control
SP600125
UO126
SB203580
A549
ATF3
actin
MCF-7
ATF3
actin
PC3
ATF3
actin
SK-OV3
ATF3
actin


B
control
DMSO
15min TNFα
5μM M344
0
5
10
μM SB203580
ATF3
actin
p-HSP27
HSP27


C
Relative Quantitation (RQ)
15
10
5
0
control
M344
M344+ SB

eliminates the MAPKinase pathways as regulators of ATF3 induction by M344 (Figure 3.3A). Although, decreased expression of ATF3 was observed following M344 treatment in the presence of JNK inhibitor in the MCF-7 cell line and ERK inhibitor in the SKOV-3 cell line, lack of consistency between cell lines allows us to conclude that MAPKinase pathways are likely not involved in mediating ATF3 induction by M344. In contrast, the ERK pathway inhibitor, UO126, could increase ATF3 expression when treated in combination with M344 on the A549 and PC3 cell lines (Figure 3.3A). Since ATF3 is a known stress inducible gene, the combination of M344 and inhibition of the ERK pathway, whose function is to mediate cell growth and differentiation, may specifically induce higher levels of ATF3 as a stress responsive cellular event. Of note in these cell lines, the inhibitors tested consistently inhibited ATF3 induction by cisplatin indicating a role for these MAPKinase cascades in cisplatin [19.3] but not M344 induction of ATF3 expression.

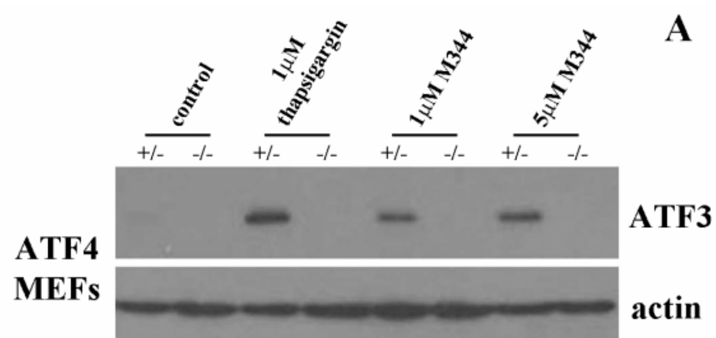
To rule out the involvement of the p38 MAPKinase pathway which we had previously shown had the most significant role in ATF3 induction by cisplatin, we more rigorously analyzed the role of the p38 MAPKinase pathway in M344 induction of ATF3. To determine the involvement of the pathway in mediating M344 induction of ATF3 the p38 specific inhibitor, SB203580 (SB), was utilized at increasing doses in the presence of M344 treatment for 24 hrs in the MCF-7 cell line. The pathway was effectively down regulated following inhibitor treatment in a dose dependent manner as measured by the phosphorylation status of heat shock protein 27 (HSP27), a downstream effector of the p38 pathway, however ATF3 expression was unaffected (Figure 3.3B). Controls included no treatment, DMSO was used as a control for the M344 vehicle, and TNFα as a positive

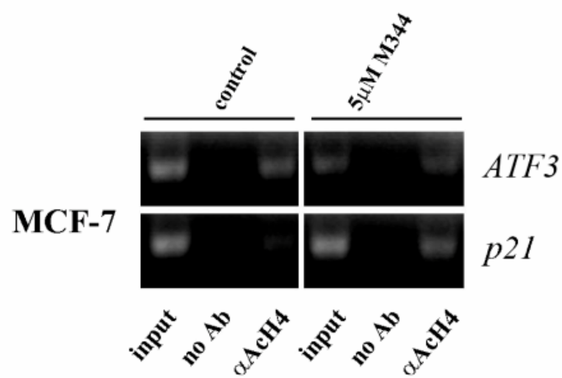
control for p38 activation. To confirm this observation we also determined the mRNA expression of ATF3 following M344 treatment in the absence and presence of the p38 expression between treatments (Figure 3.3C). Taken together, these data confirm a MAPKinase independent mechanism as a mediator of ATF3 induction by M344.

Previously our laboratory had identified lovastatin, a potent inhibitor of mevalonate synthesis, as an inducer of the ISR pathway and subsequent mediator of lovastatin-induced apoptosis [34.3]. Downstream effectors of the ISR pathway include members of the ATF family of transcription factors, ATF4 and its downstream target ATF3 [31.3]. Therefore, we looked at the potential involvement of the ISR pathway, and specifically ATF4, as a mediator of ATF3 induction by M344. We tested the ability of M344 to induce ATF3 expression in immortalized ATF4 heterozygous (+/-) or null (-/-) MEFs, the upstream inducer of ATF3 expression in the ISR pathway. Using thapsigargin, a well established inducer of the ISR, as a positive control [36.3], we show in Figure 4.3A that the absence of ATF4 completely inhibits ATF3 induction by M344 revealing an ISR dependent mechanism.

Since it has been shown that HDAC inhibition can mediate induction of genes by directly influencing the acetylation of histones surrounding the gene thus promoting transcription, we performed a ChIP assay to evaluate the association between acetylated Histone 4 (AcH4) and the ATF3 promoter. Chromatin was isolated from the MCF-7, and PC3 cell lines following treatment with solvent control or M344 at 1 and 5μM doses. Chromatin- protein complexes were pulled down with an antibody against AcH4 and the DNA was assessed for the presence of the ATF3 promoter region. In both cell lines, pull down with AcH4 antibody in the untreated cells yielded the presence of the ATF3 promoter

Figure 4.3 M344 induced ATF3 is mediated through the Integrated Stress Response. A, Western blot analysis for ATF3 and actin in ATF4 (+/-) and (-/-) MEFs treated with solvent control, thapsigargin (1μM), M344 (1 and 5μM) for 24 hrs. B, Chromatin Immunoprecipication (ChIP) assay of chromatin/DNA complexes isolated from MCF-7 cells treated with solvent control or M344 (5μM) for 24 hrs using acetylated Histone 4 (AcH4) antibody and analyzing by PCR for presence of ATF3 or p21 promoter regions.


A
control
1μM thapsigargin
1μM M344
5μM M344
+/-
-/-
+/-
-/-
+/-
-/-
+/-
-/-
ATF3
ATF4
MEFs
actin

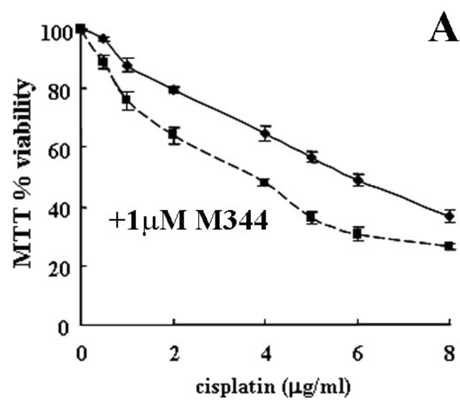

control
5μM M344
B
ATF3
MCF-7
p21
input
no Ab
αAcH4
input
no Ab
αAcH4

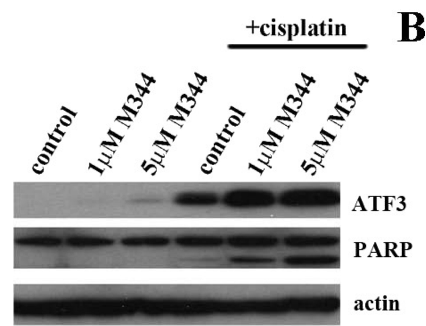
without significant enhancement with M344 treatment (Figure 4.3B). Following M344 treatment, ATF3 gene expression was increased as compared with control cells (see Figure 1), however, ATF3 promoter recovery associated with AcH4 was not increased as compared with control (Figure 4.3B) suggesting the induction of ATF3 by M344 is independent of histone acetylation association with the ATF3 gene promoter. As a control, M344 treatment induced AcH4 at the p21 promoter, a well established target of HDAC inhibition whose expression is up-regulated through promoter histone acetylation [35.3]. These data suggest the induction of ATF3 by M344 to be indirect and related to its activation and induction of effectors of the ISR.

ATF3 regulates, in part, the enhanced cytotoxicity of cisplatin and M344.

To determine whether ATF3 expression affects the enhanced cytotoxicity observed between cisplatin and HDAC inhibitor treatments, we evaluated ATF3 induction by M344 and cisplatin combination treatment in the A549 cell line. As demonstrated for the MCF-7 and SKOV-3 cells in Figure 2.3A, the combined drug treatments in A549 cells was associated with increased cytotoxicity compared to cisplatin treatment alone as analyzed by the MTT cell viability assay (Figure 5.3A). Furthermore, the combined treatment of cisplatin and M344 also resulted in enhanced ATF3 expression as compared with cisplatin and M344 alone as observed by Western blotting (Figure 5.3B). Likewise, PARP cleavage, a marker of apoptosis, was observed to increase following cisplatin and M344 treatment in combination compared with M344 and cisplatin treatment alone (Figure 5.3B). To further elucidate the role of ATF3 in enhanced cytotoxicity by HDAC inhibitors in combination with cisplatin, we expressed shRNA targeting ATF3 in the A549 cell line. To determine the role of ATF3

Figure 5.3 ATF3 mediates, in part, the enhanced cytotoxicity between M344 and cisplatin. A, A549 cells treated with cisplatin alone (0–10µg/ml), or with M344 (1µM) for 48 hrs were assessed for cell viability as measured by MTT activity. Data is represented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars represent the standard deviation of six replicates from two independent experiments where all differences in cytotoxicity observed are statistically significant to a p-value of > 0.005. B, A549 cells treated with solvent control, M344 alone (1 and 5µM), cisplatin (2µg/ml) alone or in combination with M334 (1 and 5µM) were analyzed by Western blotting for ATF3, PARP and actin.


A
100
80
60
40
20
0
MTT % viability
+1μM M344
0
2
4
6
8
cisplatin (μg/ml)

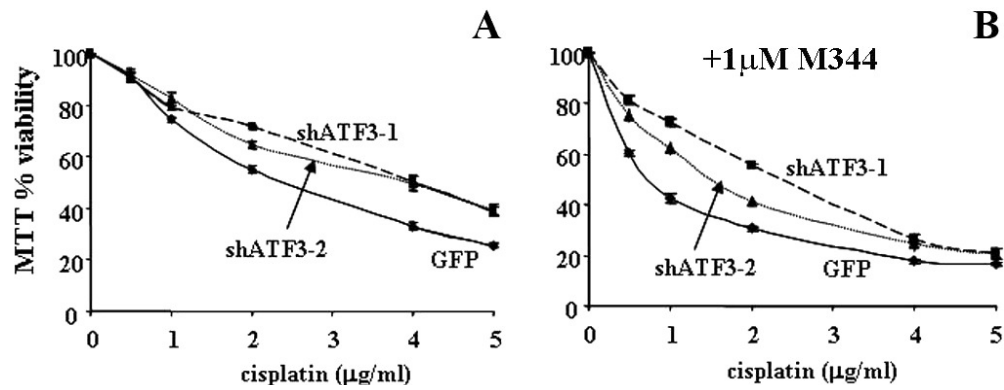

+cisplatin
B
control
1μM M344
5μM M344
control
1μM M344
5μM M344
ATF3
PARP
actin

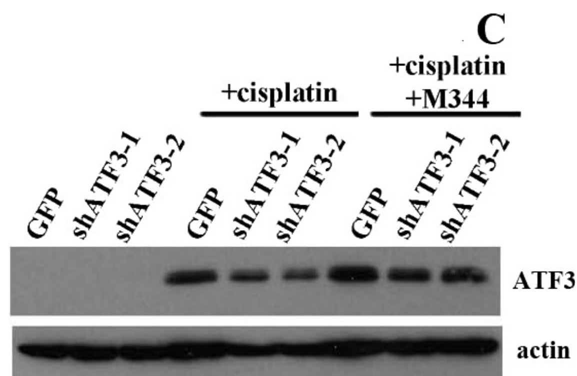
expression in drug-mediated cytotoxicity, GFP (negative control), shATF3 1 and 2 stably expressing cell lines that target two distinct sequences of the ATF3 gene were treated with cisplatin alone (Figure 6.3A) or cisplatin in combination with M344 (Figure 6.3B) and analyzed by the MTT assay. As previously reported [19.3], the shRNA expressing ATF3 targeted A549 cell lines showed attenuated cisplatin induced cytotoxicity as compared with GFP control (Figure 6.3A). M344 (1µM) treatment in combination with cisplatin enhanced cell cytotoxicity as compared with cisplatin alone in all cell lines (Figure 6.3A and 6.3B). Cytotoxicity was also attenuated in both of the shATF3 cell lines compared with GFP control when treated with cisplatin in combination with M344 (Figure 6.3B). Cisplatin (2µg/ml) and M344 (1µM) combined treatment enhanced ATF3 expression in the GFP control and shRNA targeted ATF3 A549 cell lines while ATF3 induction remained less in the shRNA targeting ATF3 A549 cells compared with control (Figure 6.3C). Since the inhibition of ATF3 expression inhibits the enhanced cytotoxicity of this drug combination, these data provide evidence that ATF3 plays a role in mediating the enhanced cytotoxic response.

Discussion

In this study, we identified ATF3 as a novel consistently inducible target of HDAC inhibitor treatment in a panel of human derived cancer cell lines both at the protein and mRNA level. Similarly in a very recent study, ATF3 was identified as one of a number of genes induced following a genetic screen of an HDAC inhibitor in sensitive colon cancer cell lines although the mechanism of induction was not characterized [37.3]. This is the first study to characterize this regulation in multiple cancer cell lines as well as address the mechanism of

Figure 6.3 Targeting ATF3 expression inhibits M344 and cisplatin co-operative cytotoxicity. A, A549 cells stably expressing GFP or shATF3-1 and shATF3-2 were treated with cisplatin (1-5µg/ml) for 48 hrs and analysed for MTT activity as above. B, A549 cells stably expressing GFP or shATF3-1 and shATF3-2 were treated with cisplatin in the presence of M334 (1µM) for 48 hrs and analysed for MTT activity as above. C, A549 cells stably expressing GFP (negative control), or short hairpin RNA against two separate ATF3 mRNA regions (shATF3-1) and (shATF3-2) were untreated, treated with cisplatin (10µg/ml) or cisplatin in combination with M344 (5µM) for 24 hrs and analyzed by Western blotting for ATF3 and actin expression. Error bars represent the standard deviation from six individual treated samples. All differences in cytotoxicity observed are statistically significant to a p-value of > 0.005.


A
B
+1μM M344
MTT % viability
100
80
60
40
20
0
shATF3-1
shATF3-2
GFP
0
1
2
3
4
5
cisplatin (μg/ml)


C
+cisplatin
+cisplatin
+M344
GFP
shATF3-1
shATF3-2
ATF3
actin

HDAC inhibition induced ATF3 expression. Regulators of ATF3 expression include the MAPKinase pathways as well as ISR activation. In M344 treatments, MAPKinase pathways, including the p38, ERK and JNK pathways, did not play a role in the induction of ATF3 expression by this HDAC inhibitor. In contrast, we have recently demonstrated that these same MAPKinase pathways regulate cisplatin induced ATF3 expression. To address the role of MAPKinases, we employed specific inhibitors to these pathways in a cancer cell line panel and found no consistent inhibition of M344 mediated ATF3 induction. Interestingly, we observed an up regulation of ATF3 expression when treating A549 and PC3 cell lines with M344 in combination with the ERK inhibitor UO126. Combination treatment of the MEK/ERK inhibitor UO126 and the HDAC inhibitor SAHA lead to increased apoptosis in leukemia cell lines [38.3], however, ATF3 levels were not assessed [38.3].

In this study, we provide evidence for the involvement of the ISR pathway as mediator of M344 induction of ATF3. M344 induced expression of ATF3 was completely abolished in ATF4 (-/-) MEFs implicating an ISR dependent mechanism downstream of ATF4. In accordance with this finding, the endoplasmic reticulum chaperone protein glucose-regulated protein 78 (GRP78) was recently identified as a non-histone target of SAHA, whose action leads to dissociation of GRP78 and its client protein, double-stranded RNA-activated protein-like ER kinase (PERK), and subsequent activation of the ISR through the induction of the endoplasmic reticulum stress response including activation of ATF4 [39.3]. Since ATF3 is a known effector of the ISR pathway downstream of ATF4 [36.3], our finding that M344 induces ATF3 may be mediated by HDAC inhibitor mediated acetylation of GRP78. Furthermore, we also demonstrated through ChIP assay of the ATF3 promoter that levels of acetylated histone H4/chromatin were independent of M344 suggesting the induction of ATF3 was not the result of increased histone acetylation at the ATF3 promoter.

A role for ATF3 in tumourigenesis has been implicated through its documented role as an apoptotic factor in cancer models, whose mechanism may be related to ATF3's role in transcriptional regulation of a number of regulators of apoptosis and cell proliferation including pro-apoptotic factor, GADD153/CHOP and cell cycle factor, cyclin D1, respectively [40.3, 41.3]. Depending on the cell type and the type and severity of the cell stressor, ATF3 has been implicated as both a proto-oncogene and tumour suppressor. Examples of ATF3 as a pro-apoptotic include an ATF3 over-expression model which lead to inhibition of proliferation and induced cell cycle arrest in human cancer cells [27.3], and loss of ATF3 in a Ras transformed model which resulted in higher proliferation rates and increased G1 to S phase transition efficiency [28.3].

As stated, HDACs catalyze the removal of acetyl groups from histones resulting in chromatin condensation and transcriptional repression [1.3, 2.3]. HDAC inhibitors reverse this transcriptional silencing of genes, including tumour suppressors [1.3, 2.3]. Coupled with their ability to induce such anti-cancer cellular processes as cell cycle arrest, apoptosis, and disruption of angiogenesis, HDAC inhibitors have been studied for their potential as cancer therapeutic agents [1.3-4.3]. Cisplatin, on the other hand, is considered a DNA-damaging anticancer drug forming different types of bi-functional adducts in reaction with cellular DNA. The final cellular outcome of DNA adduct formation is generally apoptotic cell death, thought to occur through halting of cellular processes such as replication and transcription leading to prolonged G2 phase cell-cycle arrest and deregulation of signal transduction pathways involved in growth, differentiation, and stress responses [7.3]. There is a growing body of evidence that demonstrates that HDAC inhibitors can enhance the anticancer activity of a variety of chemotherapeutic drugs, including cisplatin [12.3-17.3, 42.3].

Previous reports have attempted to identify the factors related to HDAC inhibitors ability to enhance cisplatin induced cell death including decreasing the levels of the antioxidant intracellular reduced glutathione or the involvement of the endoplasmic reticulum stress response as a mediator of the enhancement of cytotoxicity in the same cancer model [43.3]. Up-regulation of the expression by HDAC inhibitors in apoptosis associated proteins p53, BID, cytochrome c and caspase-3 have also been proposed as targets of HDAC inhibitors that can enhance cisplatin-induced cytotoxicity [16.3]. In this study we identified the transcription factor ATF3 as a mediator of enhanced cisplatin induced cytoxicity by HDAC inhibition. Identification of the specific pathway(s) of apoptotic cell death related to ATF3's role as mediator of enhanced cytotoxicity by combinational treatment merits further investigation.

Footnote:

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Chapter 4: Discussion

4.1. Cisplatin induced ATF3, via the MAPKinase pathways, plays a role in mediating its cytotoxicity

As presented in Chapter 2, this thesis demonstrates the novel finding that the clinically relevant chemotherapeutic drug, cisplatin, causes potent induction of the stress inducible gene, ATF3, in a variety of human derived cancer cell line models. ATF3 expression was also found to regulate cisplatin mediated cytotoxicity which demonstrates a novel role for ATF3 in the context of chemotherapeutic efficacy. This work, therefore, contributes to our understanding of cisplatin's ability to induce tumour cell cytotoxicity and identifies a novel factor involved in propagating this effect downstream from its classical function as a DNA damaging agent.

4.1.1. Pathways that mediate cisplatin induced cytotoxicity

A number of pathways have been identified as mediating factors in cisplatin induced cytotoxicity downstream of DNA-adduct formation. These pathways include those of adduct repair and apoptosis. It has been shown that cisplatin treatment leads to the initiation of adduct repair pathway activity and that increased expression of repair pathway factors, such as NER pathway factor ERCC-1, correlates with cisplatin resistance (21-23). Therefore, DNA repair pathways successful in the removal of cisplatin induced DNA adducts have been suggested to a play a role in tumour cell chemodrug resistance. Similarly, the apoptotic response has also been implicated in cisplatin cytotoxic outcomes. The tumour suppressor, p53, classically mutated in a number of human cancers, mediates a number of cellular responses including cell cycle progression and apoptosis through its

function as a transcription factor and regulator of cell cycle and apoptotic factors, p21 and Bcl-2 family members, respectively (33-35). P53 has been shown to play a role in cisplatin induced cytotoxicity which mechanistically is thought to occur through its central role in mediating cell cycle arrest and pro-apoptotic outcomes (3, 44). Other influencial factors shown to correlate with cisplatin mediated cytotoxicity include, caspases, the ratio between anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax, and ROS generation (2).

Besides DNA repair and classical apoptotic pathways, the MAPKinase pathways have also been shown to induce as well as correlate with cell death outcomes following cisplatin treatment in models of tumourgenesis.

4.1.1.2. Upstream factors mediating cisplatin induction of the MAPKinase pathways

A number of studies have shown a link between cisplatin and the MAPKinase pathways in regulating the anti-cancer properties of the DNA damaging drug (78). Our understanding of how cisplatin induced DNA damage leads to activation of MAPKinase pathways remains limited. It is also important to note that although generally accepted that the anti-cancer function of cisplatin pretains to DNA adduct formation, cisplatin adduct formation is not limited to DNA and has been shown to react with thiol-containing proteins (7, 11, 12), cytoskeletal microfilaments (198, 199), and RNA which may also factor into MAPKinase pathway activation (2, 200). A number of factors mediating cisplatin induced activation of the MAPKinase pathways have been proposed. Members of the Ras, GTPase signalling molecules, superfamily such as Ras (KRAS) and Kinase suppressor of Ras-1 (KSR1) have been implicated in mediating cisplatin induction of the ERK pathway. Woessmann et al. show that ERK pathway activation by cisplatin could be completely inhibited by transient expression of dominant negative Ras-N117 in an oseosarcoma cell line

(201). Likewise, Kim et al. report that loss of kinase supressor of Ras-1 (KSR1), an established positive regulator of Ras signalling cascade as well as MAPKinase ERK pathway activity, in KO MEFs correlated with decreased cisplatin induced ERK pathway activation and increased resistance to cisplatin induced apoptosis (202). Reconstitution of KSR1 in KSR1 null MEFs lead to increased ERK pathway activity and increased cisplatin sensitivity (202). Both groups also show ERK pathway inhibition, utilizing the inhibitor UO126, resulted in enhanced resistance to cisplatin, which is in accordance with results presented in Chapter 2 (Figure 6.2). Protein kinase C (PKC) which functions in receptor desenitization, transcriptional regulation, immune response and cell growth cellular processes (203), has also been shown to act upstream of cisplatin induced MAPKinase pathway activation. Basu et al. show that inhibition of PKCΔ with specific inhibitors or by siRNA knockdown both resulted in inhibition of cisplatin induced ERK pathway activation (204). In constrast, Baldwin et al. show that PKCι could negatively regulate cisplatin induced p38 pathway activity in a glioblastoma cell model and thus limit cisplatin cytotoxcity (205). These reports provide evidence for a role of PKC in regulating the activity of MAPKinase pathways upstream of cisplatin induction. The non-receptor tyrosine kinase c-Abl, activated downstream of the ATM DNA damage response (50), has also been implicated in cisplatin mediated MAPKinase pathway activation. Kharbanda et al. show that c-Abl is activated by cisplatin and mediates JNK/SAPK pathway activity through direct interaction and phosphorylation of upstream MEK kinase (MEKK1) (206). As previously discussed, p53 status as well as ROS generation have been implicated in mediating cisplatin induced cytotoxicity. Bragado et al. show that p53 mediated ROS generation act upstream of p38 MAPKinase pathway activation following cisplatin treatment in a colon carcinoma cell line

(60). The group proposes all three factors (p53/ROS/p38) are required for cisplatin induced apoptosis (60). Recently, the transcription factor Twist, associated with epithelial-mesenchymal transition (EMT) and enhancement of cancer cell invasiveness and metastatic capacity, was shown to regulate MAPKinase pathway activity following cisplatin treatment (207). Zhuo et al. report that depletion of Twist with siRNA resulted in increased JNK pathway activity and sensitization to cisplatin treatment in a lung carcinoma cell line (207), implicating Twist in cisplatin mediated MAPKinase regulation. Taken together, a number of diverse regulatory factors have been identified upstream of cisplatin induced MAPKinase pathway activation.

4.1.1.3. Factors downstream of cisplatin induced MAPKinase pathways

This thesis identifies the pro-apoptotic factor, ATF3, as a downstream effector to cisplatin induced MAPKinase pathways as well as a mediator of cisplatin induced cytotoxicity. Other groups have proposed factors downstream to cisplatin/MAPKinase pathway activation that influence cisplatin induced cytotoxicity. These factors include those involved in the apoptotic pathway, cell cycle regulation, and known for tumour suppressor function. In terms of apoptotic factors, FasL and pro-apoptotic Bcl-2 members Bak and Bad, have been implicated downstream of cisplatin induced MAPKinase pathway activation. Mansouri et al. show that cisplatin induced JNK and p38 pathway activation was required for cisplatin induced cytotoxicity and JNK pathway activation was associated with hyperphosphorylation of c-Jun and downstream target expression of the extrinsic pathway factor, FasL in a cisplatin sensitive ovarian carcinoma cell line (74). Furthermore, inhibition of FasL function with neutralizing antibodies blocked cisplatin induced cytotoxicity suggesting a regulatory role for the factor downstream of MAPKinase pathway

activation (74). Similarly, Mandic et al. report the involvement of Bak, known for its role in the regulation of mitochondrial pore formation and propagation of the intrinsic pathway response, in cisplatin induced apoptosis downstream of the kinase fragment of MEKK1 (ΔMEKK1) (208). Mandic et al. show that expression of a kinase inactive fragment of MEKK1 (dominant negative ΔMEKK1) lead to inhibition of cisplatin induced Bak activation and cytochrome c release as well as, a reduction in apoptotic outcomes (208). Hayakawa et al. show that the ERK pathway mediates Bad phosphorylation upon cisplatin treatment and inhibition of this pathway leads to increased sensitivity to cisplatin in an ovarian cancer cell model (209). Taken together these reports provide evidence for the regulation of cisplatin induced cytotoxicity by apoptotic factors acting downstream of MAPKinase pathways.

Cell cycle regulators have also been implicated in cisplatin induced cytotoxicity downstream of MAPKinase pathways. DeHaan et al. show that cisplatin induced ERK activation is upstream of p21 and Gadd45 expression and that inhibition of the ERK pathway with a specific inhibitor reduces the induction of p21 and Gadd45 and increases apoptosis in an ovarian carcinoma cell model (210). The tumour supressors p53 and p73, have been reported to influence cisplatin induced cytotoxicity downstream of MAPKinase pathways. Toh et al. shows that c-Jun mediates p73 expression and activity and loss of c-Jun expression is associated with cisplatin resistance (211). Reconstitution of c-Jun into null cells restores p73 activity and sensitization to cisplatin identifying p73 downstream to cisplatin cytotoxicity outcomes (211). Sanchez-Prieto et al. report that cisplatin induction of p53 is mediated by the p38 pathway (79). The group shows direct interaction between p38 and p53 which facilitates its activity and inhibition of the p38 pathway results in

reduced cisplatin induced apoptosis (79). It is important to note, that not all reports correlate MAPKinase pathway activation with cisplatin induced pro-apoptotic outcomes. Some show a pro-survival role of MAPKinase signalling correlated with cisplatin resistance (205, 209, 210). Discrepancies have been suggested to be related to the ability of the MAPKinase pathway downstream signalling to promote DNA repair and survival (78). Our results are in accordance with a pro-apoptotic role for all three MAPKinase pathways in relation to cisplatin cytotoxicity (Chapter 2, Figure 6.2).

4.1.2. MAPKinase Pathways and ATF3 induction

In this study we demonstrate that ATF3 induction by cisplatin is mediated by the MAPKinase signalling pathways. A number of studies have shown that induction of ATF3, induced by a variety of stress inducing insults, is regulated by the MAPKinase cascades. However, the specific pathways responsible for ATF3 induction by varying stress stimuli differ among the literature. For example, an earlier report by Cai et al. show that the SAPK/JNK and not the p38 or ERK pathways is involved in ATF3 induction by homocysteine (145), Lu et al. show that the p38, not JNK or ERK pathways, is involved in anisomycin induction of ATF3 (124), and Mayer et al. report the involvement of both ERK and SAPK/JNK pathways in induction of ATF3 through the stimulation of GnRH receptors (151). Similar to our data which shows all three MAPKinase pathways are implicated in ATF3 induction by cisplatin, a very recent study reports the involvement of all three MAPKinase pathways in the induction of ATF3 in addition to apoptotic effects elicited by the anti-inflammatory drug Tolfenamic acid (147). Inoue et al. report a bidirectional regulation of ATF3 by TNF-α that requires the SAPK/JNK pathway for ATF3 induction and

the ERK pathway for ATF3 suppression (146). In contrast to our results, Hamdi et al. recently showed that cisplatin could induce ATF3 expression in a glioblastoma cell line that was dependent on the JNK, not the p38 or ERK pathways, and that reduced expression of ATF3 was associated with enhanced cisplatin cytotoxicity (212). Discrepancies in ATF3's role as a pro- or anti-apoptotic factor in relation to cancer models has been proposed to be tissue/cell line dependent or determined by the malignancy state of the model used (83, 88). What was not addressed in our study of MAPKinase pathway dependent cisplatin induction of ATF3, and remains future work, was the mechanism by which ATF3 induction is mediated by the MAPKinase cascades. Specifically what downstream transcriptional factor(s) regulated by the MAPKinase pathways is/are responsible for ATF3 gene expression. Studies have identified downstream effectors of the MAPKinase pathways as regulators of stress induced ATF3. For example, Cai et al. show that a complex containing ATF2 and c-Jun, downstream effectors to the SAPK/JNK pathway, bind to the ATF3 promoter and increase its activity (145). Mayer et al. show that ATF2 and CREB regulate GNRH receptor agonist mediated ATF3 induction through binding to the ATF3 promoter in a gonadotroph cell line (151). Lee et al. show that TA induced ATF3 expression and mediated apoptosis is dependent on ATF2 whose expression correlates with ATF3 induction, and binds to and enhances ATF3 promoter activity (147). Lu et al. also shows that CREB is involved in p38 pathway dependent anisomycin induction of ATF3 (124). Further work is needed to identify the specific downstream effects of the MAPKinase pathways which mediate cisplatin induction of ATF3.

4.2. ATF3's role in the enhanced cytotoxicity induced by HDACi and cisplatin combinational treatment

HDACis are emerging as compounds known for their anti-cancer properties which, in general, are believed to be attributed to their ability to reverse epigenetic gene silencing in the context of tumourgenesis (152, 153). The ability of tumour cells to silence genes such as tumour suppressors and effectors to apoptotic pathways proves advantageous towards cancer progression. HDACis act to promote gene transcription through inhibition of histone deacetylases which remove acetylation groups from histones resulting in chromatin condensation and transcriptional repression (152, 153). It is also important to note that HDACi activity can extend to non-histone proteins which may also contribute to their anti-cancer properties (152, 157). In this study, presented in detail in Chapter 3, we demonstrate that ATF3 is upregulated by HDACi treatment in a panel of human cancer cell lines. We show that this induction of ATF3 by HDACi was independent of its role as mediator of histone acetylation specific to the ATF3 promoter. We did however find that HDACi induced ATF3 was reliant on ATF4 which is known as a direct regulator of ATF3 expression as part of the ISR pathway. We also show that HDACi in combination with cisplatin resulted in enhanced induction of ATF3 protein and mRNA which correlated with increased cytotoxicity by the two drugs. Although other reports have shown the potential for enhanced cytotoxicity by the combination of HDACi and cisplatin we are the first to show an involvement for the pro-apoptotic factor ATF3 in mediating this effect. We are also one of the first to report the stress inducible gene, ATF3 as target influenced by HDACi.

4.2.1. HDAC inhibitor activity, ATF3 and the ISR pathway

The link between HDACi and ATF3 induction is novel and besides our study only one other very recent report identifies ATF3 as an inducible target of HDACi treatment through microarray analysis which was confirmed at the protein and mRNA level in a colon cancer cell line (213). Our report, however, is the first to characterize the mechanism of HDACi mediated ATF3 induction and to correlate ATF3 expression, induced by HDACi and cisplatin combinational treatment, with cytotoxic outcomes. We show that HDACi induction of ATF3 was independent of the MAPKinase pathways, which we previously show is responsible for cisplatin induction of ATF3. We also show that induction of ATF3 by HDACi is independent of its function as mediator of histone acetylation directly at the ATF3 promoter (Chapter 3, Figure 4.3) however, these data do not rule out an indirect regulatory role mediated by HDACi chromatin remodelling. We did find that the presence of ATF4 was required for HDACi induction of ATF3 which suggests the involvement of the ISR pathway since ATF4 is known as a direct effector of ATF3 expression following ER stress (97). Until very recently no link between HDACi and the ISR pathway had been made. Baumeister et al. report the involvement of HDAC1 and the gene regulation of the ISR chaperone protein, Grp78/Bip (214). Upon ER stress, Grp78/Bip, a protein chaperone, is induced as part of the ISR signalling cascades which functions to arrest general translation and degrade misfolded proteins (215). Baumeister et al. show that HDAC1 directly binds to the Grp78/Bip promoter and results in its gene repression, whereas HDACi treatment leads to upregulation of Grp78/Bip expression (214). Activation of the ISR can conclude with either cell survival or apoptotic outcomes which is dependent upon the severity of cellular stress and the cell's ability to overcome the insult (91). Baumeister report that HDACi treatment resulted in the induction of Grp78/Bip expression however, this upregulation was

not associated with the induction of downstream effectors such as CHOP, typically associated with the pro-apoptotic branch of the ISR pathway (214). The group correlates the induced expression of Grp78/Bip with resistance to HDACi-induced apoptosis therefore characterizing Grp78/Bip as an anti-apoptotic factor (214). This outcome is in accordance with failure to induce the apoptotic branch of the ISR pathway, namely CHOP. In contrast, our data suggests that induction of ATF3 by HDACi, downstream of the ISR, contributes to pro-apoptotic effects when combined with cisplatin.

The literature has provided, very recently, a specific mechanism by which HDACi may mediate ATF3 induction as part of the ISR pathway. Kahali et al. show that Grp78/Bip is a direct non-histone substrate of HDACi whose increased aceytlation was confirmed by mass spectrometry analysis and immunoprecipation (216). Kahali et al. show that increased acetylation of Grp78/Bip by HDACi treatment is accompanied by disassociation between Grp78/Bip and client protein, PERK (216). HDACi treatment was also shown to be associated with activation of PERK, and downstream ISR pathway effectors such as eIF2α, ATF4 and CHOP, as well as a reduction in the rate of protein synthesis (216). ATF3 was not assessed, but is known to be activated downstream of ATF4 and upstream of CHOP as part of the ISR pathway (97). Knockdown of PERK under HDACi conditions resulted in increased survival suggesting a pro-apoptotic role for this HDACi-induced cascade (216). This report is in accordance with our findings that ATF3 is induced by HDACi, as mediated by ATF4 which functions as part of the ISR, and associated with pro-apoptotic outcomes. This report also provides a specific mechanism by which HDACi induces ATF3 which may be through the induction of the ISR pathway mediated through the newly identified non-histone HDACi target, Grp78/Bip. Kahali et al. suggest that HDACi induced acetylation of

Grp78/Bip may result in disassociation of Grp78/Bip from PERK thus initiating the ISR and induction of downstream effectors of the pathway such as ATF3. Whether or not the acetylation status of Grp78/Bip affects the ability of HDACis to induce ATF3 merits further investigation.

4.2.2. Mechanisms of enhanced anti-cancer effects by HDACi in combination with cisplatin

It is well established that HDAC inhibitors can enhance the anticancer activity of cisplatin in vitro in a variety of cancer cell models (217-222). Few studies exist, however, detailing the mechanism of enhanced anti-cancer effects by HDAC inhibitors in combination with cisplatin. Rikiishi et al, correlated enhanced cytotoxicity by HDAC inhibitors in combination with cisplatin with reduced levels of the antioxidant intracellular reduced glutathione (GSH) in an oral squamous cell carcinoma (ORCC) model (223). The same group has also proposed the involvement of the ER stress response as a mediator of the enhancement of cytotoxicity in the same cancer model through results showing that increased ISR pathway activation leads to decreased cytotoxicity by the duel drug treatement (224). Shen et al. observed an upregulation in apoptosis associated protein p53, BID, cytochrome c and caspase-3 in the ORCC model following combinational treatment (221). HDAC inhibitors were observed to potentiate both the phosphorylation of histone H2AX on serine-139, a marker of DNA double-strand breaks, as well as the accumulation of ROS in an ovarian model (220). Likewise, upregulation of ROS as well as, phosphatase and tensin homolog (PTEN) by valproic acid and cisplatin was reported in an ovarian cancer model (219). Bid protein processing in truncated t-Bid and cytochrome c release from mitochondria were also observed to be significantly increased in the presence of valproate in combination with cisplatin and pemetrexed in a malignant mesothelioma model (222). Our

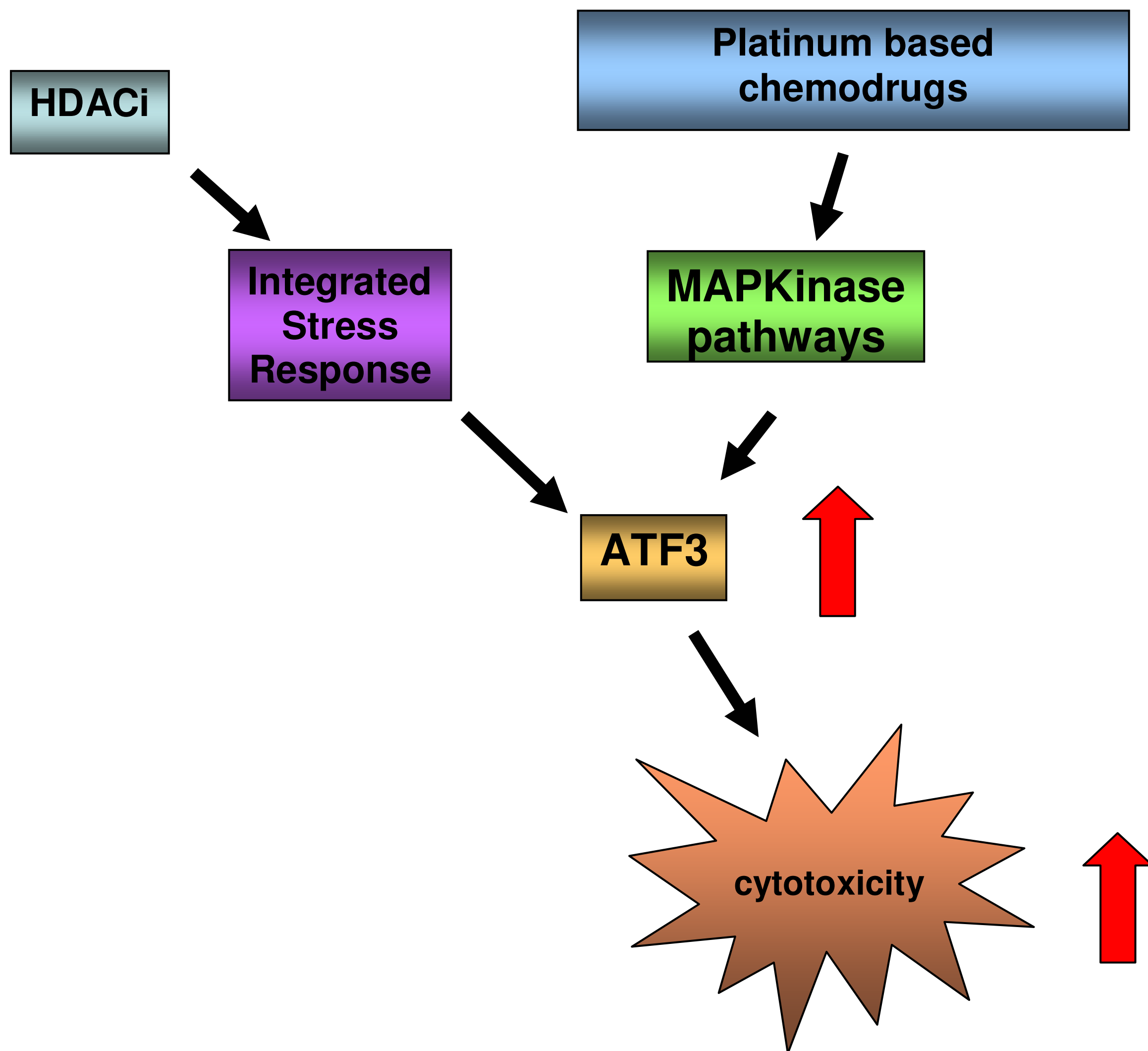
data contributes to the knowledge of what is known downstream of HDACi and cisplatin combinational treatment correlating with their enhanced anti-cancer effects. We show the pro-apoptotic factor, ATF3, to be a novel downstream effector implicated in HDACi and cisplatin cytotoxic synergism.

4.3. Future Directions and Conclusions

4.3.1. Chemotherapeutic drugs induce ATF3

This thesis characterizes the induction of ATF3 by two separate cancer chemotherapeutic drugs, cisplatin and HDACi, through very different anti-cancer cellular mechanisms. Interestingly, both of these drugs converge to induce ATF3, which was shown in this study to correlate with increased cytotoxicity by cisplatin alone (Chapter 2), or in combination with HDACi (Chapter 3). Even more intriguing is the diversity in cellular mechanisms by which these two separate drugs are capable of inducing ATF3. Cisplatin regulates ATF3 induction via the MAPKinase pathways and HDACi mediates ATF3 induction through the ISR factor ATF4 (Figure 1.4). Future directions include elucidating the specific factor(s) downstream of cisplatin and the MAPKinase pathways which leads to ATF3 induction. Future aims also include further defining the ISR as a mediator in HDACi regulated ATF3 induction and determining the role GRp78/Bip plays in propagating this induction. Understanding the specific mechanisms involved in chemodrug anti-cancer activities may lead to increased drug efficacy and survival in clinical settings.

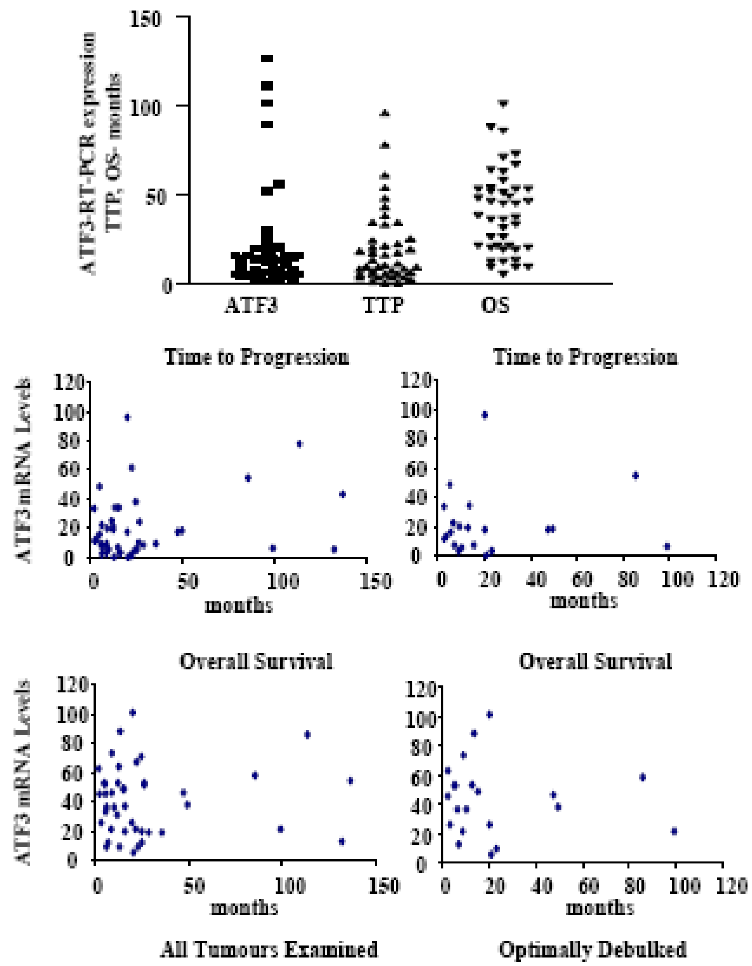
Figure 1.4 Cisplatin and HDACi combinational treatment: convergence on ATF3. Schematic depicting the induction of ATF3 by both cisplatin and HDACi treatment and the differential pathways involved.


HDACi
Platinum based chemodrugs
Integrated Stress Response
MAPKinase pathways
ATF3
cytotoxicity

4.3.2. ATF3 as a biomarker for cisplatin's clinical efficacy

Although ATF3's expression has been extensively shown to regulate survival outcomes in the context of cancer in both human cancer cell lines in vitro and mouse models in vivo, ATF3 has yet to be characterized as a biomarker in a clinical setting. Only one report to date has examined ATF3 expression in human tumour samples (88). Interestingly, ATF3 is localized to the chromosome 1q amplicon which has been identified as one of the most frequently amplified regions in solid tumours, and the most amplifed region in breast tumours (88, 225). Based on this Yin et al. analyzed the expression of ATF3 in a cohort of breast tumour samples (88). These breast cancer derived tumour samples were organized into two groups, one with elevated ATF3 expression and one with no ATF3 expression. The two groups were analyzed for gene copy number and the group with elevated ATF3 levels was shown to have a higher incidence of abnormal gene copy number ($\geq$ 3; 91%) compared with the group with non-elevated ATF3 levels which had a lower incidence of gene abberation ($\geq$ 3; 68 %) (88). It was also noted that the group with non-elevated ATF3 levels had a distribution toward low copy number (3-5) compared with the elevated ATF3 group which had a distribution toward high copy number (> 5) (88). These data, therefore, imply an oncogenic role for ATF3 in regards to frequency in genetic abberation in a human cancer model. These data are in contrast to our results which define ATF3 as a pro-apoptotic factor. Our laboratory has also characterized ATF3 levels in a cohort of human ovarian tumour samples (Figure 2.4, unpublished). Looking at ATF3 mRNA levels in these ovarian tumours (n=50), similar to Yin et al. results, we found a varied distribution of ATF3 expression levels with some highly elevated levels and some low. Attempts at correlating ATF3 levels with time to progression (TTP) and overall survival (OS) within this ovarian tumour cohort

Figure 2.4 ATF3 mRNA expression levels in human ovarian tumour samples. Levels of *ATF3* mRNA were determined in 50 ovarian carcinoma tumours by real time quantitative RT-PCR. The values for each tumour were determined through the evaluation of three independent sampling done in triplicate and the expression determined through averaging these results. The ovarian cell line A2780s treated with 2µg/ml cisplatin for 24hrs was used to standardize the results and was given a value of 1. Fold differences in expression were calculated following normalization to GAPDH levels. Graphs depicting ATF3 expression in relation to OS and TTP for all patients optimally debulked (residual disease after surgery <2cm) where n=22 are also presented. No statistically significant association was demonstrated with respect to ATF3 expression and TTP or OS in this cohort. Cox Proportional Hazards models were used to determine the relationship between OS and TTP across ATF3 level.



ATF3-RT-PCR expression TTP, OS- months
150
100
50
0
ATF3
TTP
OS
Time to Progression
Time to Progression
ATF3 mRNA Levels
months
months
Overall Survival
Overall Survival
ATF3 mRNA Levels
months
months
All Tumours Examined
Optimally Debulked

proved statistically insignificant which may reflect low sample number. Future aims would be to increase the sample number to see if ATF3 expression levels do indeed positively correlate with greater overall survival. Specific clinical analysis would be to collect tumour samples directly after a regimen of cisplatin to see whether ATF3 is induced in the tumour following drug treatment and whether ATF3 expression levels can be associated with increased efficacy of the drug. This type of clinical analysis could also be extended to dual drug treatment with cisplatin and HDACi with the same clinical aims. Overall, ATF3 provides a potentially exciting cancer biomarker and based on our data, may provide a predictive tool in the context of present day chemotherapeutic drug efficacy.

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CURRICULUM VITAE

Carly St. Germain

OBJECTIVE

Professional employment with a biochemical research and development organization.

QUALIFICATIONS

Technical

Proteomics / Cell Biology

- Practiced in both immunohistochemistry fluorescent microscopy and immunofluorescent confocal laser microscopy.
- Skilled in cell culture work.
- Highly skilled in Western Blot protein analysis in both in vivo and in vitro systems.
- Experienced in protein profiling and data analysis using Ciphergen ProteinChip technology and software.
- Cellular fractionation ultracentrifuge techniques
- Transmission Electron Microscope operation and image analysis.
- MTT cell viability assay
- Flow cytometry

Lipid Biochemistry

- Radio-labeling of cellular lipid moieties with ^{3}H-Glycerol.
- Skilled in cellular lipid extraction and both Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) analysis of cellular lipid profiles.

Molecular Biology

- Acquired skills in recombination, and purification of DNA as part of vector construction.
- Microbiology experience acquired through transformation and isolation of DNA in *E. Coli* bacterial system.
- Experience in DNA transfection into human cell lines.
- Acquired skills in animal handling protocols, DNA extraction from animal tissues and characterization of DNA for the purpose of genotyping.
- RNA cellular extraction and RT-PCR for gene expression analysis.

Reproduction

- Trained in a number of reproductive-related laboratory techniques such as mouse handling procedures, surgical oviduct removal and mouse embryo culture.

Other

- Advanced writing skills achieved through the preparation of research reports, manuscripts, abstracts, and theses.
- Excellent communication and presentation skills acquired through presentation of research and scientific journals as part of group seminar series, weekly meetings and scientific conference attendance.
- Trained in conducting data analysis, extensive scientific literature reviews, critical analysis of scientific literature, with an exceptional understanding of scientific jargon and concepts of experimental design.
- Independent worker with outstanding organization skills.

EDUCATION

- Enrolled in the PhD Biochemistry program with the University of Ottawa, January 2005 – Present (expected date of completion – early 2011)
- M.Sc. Biochemistry, University of Ottawa, 2002-2004 (The Ottawa Hospital Research Institute (OHRI))
- B.Sc. Honours Biochemistry, Bishop's University, 1999-2002 (Academic Honour Roll: 1999-2002)

LABORATORY RESEARCH HISTORY

- **PhD Research**, **Centre for Cancer Therapeutics the Ottawa Hospital Research Institute (OHRI), 2008-Present**. Exploration of Mechanistic Induction of Activating Transcription Factor 3 (ATF3); Implications in cancer therapeutics
- **PhD Research, Ottawa Heart Institute (OHI), Lipids and Athreosclerosis Group,University of Ottawa, 2005- 2008.** Studies on mechanisms by which EPA (fish oil) accelerates the rate of triacylglyceride turnover in a rat liver cellular model.
- **M.Sc. Research, Ottawa Hospital Research Institute (OHRI), Diseases of Ageing Group, University of Ottawa, 2002-2004**. Studying the expression of Proprotein Convertase 1 (PC1) in mouse preimplantation embryos and determining the mechanism of transient translocation to the nuclei in one cell fertilized embryos.
- **Summer Student, Ottawa Health Research Institute (OHRI), Diseases of Ageing Group, 2002.** Gained experience on Ciphergen ProteinChip Array technology as a main user for the Diseases of Ageing Group by conducting protein profiling and immunocapture assay experiments on cultured media from human *in vitro* egg cultures.

POSTER/ PRESENTATION / PUBLICATIONS

Poster, Conference Attendance, and Oral Presentation:

- **C. St. Germain**, L. Ma, K. Garburio, A. O'Brian, and J. Dimitroulakos. The role of Activating Trancription Factor 3 (ATF3) in chemotherapeutic induced cytotoxicity. Presented as a poster at the Annual OHRI Research Day (University of Ottawa, 2008, 2009) and Annual University of Ottawa BMI Poster Day (2009).
- Attended the first annual FASEB Summer Research Conference: 'Lipid Droplets: Metabolic Consequences of the Storage of Neutral Lipids' (Saxtons River Vermont, 2007).
- Attended the 46th annual American Society of Cell Biology Meeting (San Diego, California, 2006) appearing as co-author on the poster: W.Y. Yang, G. Thorne-Tjomsland, **C. St. Germain**, K. Tran, X. Xie, J. Jamieson, and Z. Yao. Polyunsaturated Omega-3 Fatty Acid Mediates Hepatic Lysosomal-degradative Activities Towards Intracellular Triglycerides.
- **C. St.Germain**, and Z. Yao. Hepatic Autophagy Induced by n-3 Fatty Acids and Implications in Lipid Metabolism (2005). Presented orally at University of Ottawa Heart Institute Research Day (2005) and as a poster at the Annual University of Ottawa BMI Poster Day (2007).
- **C. St.Germain**, G. Croissandeau, J. Mayne, J. Baltz, M. Chrétien and M. Mbikay. Expression and Transient Nuclear Translocation of Proprotein Convertase 1 (PC1) During Mouse Preimplantation Embryonic Development (2004). Presented as a poster at the Canadian Workshop on Human Reproduction and Reproductive Biology (Ottawa, 2004) and the Annual OHRI Research Day (2004).
- **C. St. Germain**, J. Mayne, M.C. Léveillé, and M. Mbikay. Biomarker Patterning During In Vitro Human Embryonic Development (2003). Presented as a poster at the Annual OHRI Research Day (2003) and Ottawa Reproductive Biology Workshop (2003) and orally at Retreat for the Diseases of Ageing and Biochemical Neuroendocrinology Clinical Research Groups (Ottawa, 2002).

Publications

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

- **C. St. Germain**, N. Niknejad, L. Ma, K. Garbuio, T. Hai and J. Dimitroulakos. Cisplatin induces cytotoxicity via the mitogen-activated protein kinase pathways and Activating Transcription Factor 3. (2010) Neoplasia. 7, 527-38.

- **C. St. Germain**, W.Y. Yang, P. Rippstein, X. Sunney Xie, and Z. Yao. N-3 fatty acid treatment enhances the turnover of hepatic triacylglycerol in addition to lowering triacylglycerol secretion. (2008) Pending submission.

- M. Pan, V. Maitin, S. Parathath, U. Andreo, S.X. Lin, **C. St. Germain**, Z. Yao, F.R. Maxfield, K.J. Williams, E.A. Fisher. Presecretory oxidation, aggregation, and autophagic destruction of apoprotein-B: a pathway for late-stage quality control. (2008) Proc. Natl. Acad. Sci. U S A. 105, 5862-7.

- K. Tran, F. Sun, Z. Cui, G.Thorne-Tjomsland, **C. St. Germain**, L. R. Lapierre, R. S. McLeod, J. C. Jamieson, and Z. Yao. Attenuated secretion of very low density lipoproteins from McA-RH7777 cells treated with eicosapentaenoic acid is associated with impaired utilization of triacylglycerol synthesized via phospholipid remodeling. (2006) BBA. 4, 463-73.

- **C. St. Germain**, G. Croissandeau, J. Mayne, J. Baltz, M. Chrétien and M. Mbikay. Expression and Transient Nuclear Translocation of Proprotein Convertase 1 (PC1) During Mouse Preimplantation Embryonic Development. (2005) Mol. Reprod. Dev. 72, 483-493.

COMPUTER SKILLS AND TRAINING

- **General**: Microsoft Office (Excel, PowerPoint, Word) and Reference Manager.
- **Biological Science–Related**: Ciphergen ProteinChip Software, Confocal System BioRad (Ver. 3.2), Zeiss Axiovision 2.05, ImageJ 1.32j, PAWS, Gene Construction Kit 2.5, PUBMED, QuantityOne (BioRad)
- **Training**: Workplace Hazardous Material Information System, Ottawa Hospital Radiation Safety Training.

AWARDS RECEIVED

- Ontario Graduate Scholarships in Science and Technology (OGSST)
  $15,000/year (2005-2007)
- Biochemistry Departmental Entrance Scholarship from the University of Ottawa
  $4000/year (2002-2004)
- H. Greville Smith Memorial Scholarship in Science from Bishop's University
  $2000/year (1999-2002)

LANGUAGES

- English
- French (Working Knowledge)

REFERENCES

- Available Upon Request