The Role of Activating Transcription Factor 3 as a Regulator of DNA-Damaging Chemotherapy Cytotoxicity

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Abstract

DNA-damaging chemotherapeutics are a consistently employed for the treatment of cancer, and are regularly part of first-line combination therapeutic regimens. However, these regimens often display limited activity in cancers including of the lung and breast. Novel therapeutic strategies are urgently required. Understanding the molecular mechanisms regulating DNA-damaging chemotherapeutics activity will lead to such novel strategies.

Activating transcription factor 3 (ATF3) is a stress inducible gene that plays a significant role in regulating cellular stress including DNA damage. This thesis investigates the role of ATF3 in mediating the cytotoxic effects of two commonly employed DNA-damaging chemotherapeutics, cisplatin and doxorubicin. This study also identifies other independent ATF3 inducing agents in potential novel combination therapeutic strategies.

In this study, cell line models of cisplatin-resistance were generated independently from two non-small cell lung cancer (NSCLC) cell lines, Calu6 and H23. Full transcriptome RNA-sequencing analysis identified ATF3 as the most highly dysregulated apoptosis regulatory gene in the cisplatin-resistant compared to their respective parental cell lines following treatment with cisplatin. Further characterization identified cisplatin induced activation of JNK as the key regulator of ATF3 induction in these cell lines. Restoring JNK activity resulted in induced ATF3 expression and re-sensitization of the resistant cell lines to cisplatin treatment. FDA-approved 1200 compound library screens were employed to identify agents that can enhance cisplatin cytotoxicity as well as whose cytotoxicity was dependent on ATF3 expression. Vorinostat, an HDAC inhibitor, was identified in both screens and importantly displayed synergistic cytotoxicity in combination with cisplatin.

In addition, ATF3 was also induced with treatments of the DNA damaging agent doxorubicin in these NSCLC cell lines including in the cisplatin resistant models. This work suggested a different mechanism of ATF3 induction by doxorubicin and the potential role of ATF3 in mediating doxorubicin cytotoxicity was further investigated in breast cancer cells. Doxorubicin robustly increased ATF3 expression in human breast cancer cell lines and tumor tissue *ex-vivo*. Loss of ATF3 in MEFs resulted in reduced sensitivity to doxorubicin treatment compared to wild-type MEFs. Employing the same library screen as above, compounds with ATF3 dependent mechanisms that could enhance doxorubicin cytotoxicity were identified. Vorinostat, as well as, the nucleoside analogues trifluridine and 6-mercaptopurine induced ATF3 expression and enhanced doxorubicin cytotoxicity.

This study demonstrated that ATF3 plays an important role in mediating the cytotoxic effect of the DNA-damaging chemotherapeutics cisplatin and doxorubicin. It also provides rationale for ATF3 as a potential therapeutic target, and for the incorporation of ATF3 inducing agents in novel combination therapeutic strategies.

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List of Abbreviations

ABC	ATP-Binding Cassette
AKR	Aldo-Keto Reductase
AP1	Activating Protein 1
ATF3	Activating Transcription Factor 3
ATM	Ataxia-Telangiectasia Mutated
ATP7B	ATPase Copper Transporting Beta
ATR	Ataxia Telangiectasia and Rad3-Related Protein
Bcl-2	B-Cell Lymphoma 2
BMM	Bone Marrow Macrophage
bZIP	Basic Region-Leucine Zipper
C/EBP	CCAAT/Enhancer Binding Protein
CHOP	C/EBP Homologous Protein
CI	Combination Index
cisR	Cisplatin Resistant
CREB	cAMP Responsive Element Binding
CTR1	Copper Transporter 1
DDR	DNA-Damage Response
DMEM	Dulbecco's Modified Eagle's Medium
DNA-PK	DNA Protein Kinase
DR5	Death Receptor 5
EGR1	Early Growth Response 1
eIF2a	Eukaryotic Initiation Factor 2 alpha
ERCC1	Excision-Repair Cross Complementation Group 1
ERK	Extracellular Regulated Kinase
Fas	First Apoptosis Signal Receptor
FasL	Fas Ligand
GADD153	Growth Arrest and DNA-Damage Inducible Gene 153
GCN2	General Control Non-Derepressible 2
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylate
HDACi	HDAC inhibitors
HRI	Haem-Regulated Inhibitor

HSP	Heat Shock Protein
Id-1	Inhibitor of Differentiation 1
IL	Interleukin
ISR	Integrated Stress Response
JNK	c-JUN N-Terminal Kinase
KLF6	Kruppel-Like Factor 6
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MCP1	Monocyte Chemotactic Protein 1
MRP2	Multidrug Resistance Protein 2
NER	Nucleotide Excision Repair
NFkB	Nuclear Factor kappa B
NK	Natural Killer
NSCLC	Non-Small Cell Lung Cancer
OPRT	Orotate Phosphoribosyltransferase
OS	Overall Survival
PERK	PKR-like Endoplasmic Reticulum kinase
PFS	Progression Free Survival
P-gp	P-glycoprotein
PKR	RNA-Dependent Protein Kinase
PTEN	Phosphatase and Tensin Homolog
PUMA	p53 Upregulated Mediator of Apoptosis
ROS	Reactive Oxygen Species
STAT	Signal Transducer and Activator of Transcription
TAS-102	Trifluridine/Tipiracil
TLR	Toll-Like Receptor
TNBC	Triple negative Breast Cancer
TNFa	Tumor-Necrosis Factor alpha
TopIIa	Topoisomerase II alpha
TRAIL	Tumor-Necrosis Factor-Related Apoptosis-Inducing Ligand
UTR	Untranslated Region
XIAP	X-linked Inhibitor of Apoptosis Protein
XPC	Xeroderma Pigmentosum, Complementation Group C
XPF	DNA Repair Endonuclease XPF

Chapter 1: Introduction

1.1 Non-Small Cell Lung Cancer

Lung cancer is the leading cause of cancer related death in Canada accounting for approximately 26% of all cancer mortalities, greater than the next three prevalent cancers combined [1]. Additionally, there is a dismal overall survival rate in patients with lung cancer as the 5 year overall survival is only 17% [1]. Non-small cell lung cancer is the most common form of lung cancer comprising 85% newly diagnosed of cases, with small-cell lung cancer encompassing the remaining 15% [2, 3]. NSCLC is divided in to three histological phenotypes, adenocarcinoma (approximately 50% of cases), squamous cell carcinoma (approximately 35% of cases), and lastly large cell carcinoma (approximately 15% of cases) [2, 3]. Adenocarcinoma is more commonly diagnosed at earlier stages since it generally proliferates and grows at a slower rate than the other subtypes [4]. Adenocarcinomas of the lung are most common in never smokers and typically arise from cells on the periphery of the lung and possess glandular or secretory properties [4, 5]. Lung adenocarcinomas often express biomarkers that are consistent for cells originating from the distal lung, including thyroid transcription factor 1, and keratin 7[6]. Squamous cell carcinoma arises from glandular or secretory cells that line the epithelial airway and its incidence is more strongly associated with smoking [4, 5]. Squamous cell carcinomas are distinguished from adenocarcinomas by immunostaining for cytokeratin 5 and 6, and/or the transcription factors SRY-box 2 and p63 [6]. Lastly, large cell carcinomas tumors are phenotypically undifferentiated and lack the characteristics of the other subtypes [4, 7]. These tumors are diagnosed based on exclusion if they do not appear to be glandular or squamous in

morphology, or express biomarkers for either adeno- or squamous cell carcinomas [6]. Multiple risk factors are associated with the development of lung cancer, including environmental (smoking and pollution) and genetic factors. Smoking is considered one of the major risk factors for the development of lung cancer [8] and accounts for a large proportion of lung cancer deaths [9]. Mutations in oncogenes and tumor suppressors increase the chance of developing lung cancer regardless of exposure to environmental factors including activating mutations in the oncogene KRAS which occurs in approximately 25- 40% of lung cancers [10].

Recent advances in next-generation sequencing (NGS) have allowed for an expansive examination and determination of genetic alterations occurring within cancers and the identification of driver mutations. Along with other high-throughput genomic profiling, this has provided the ability to further characterize the molecular subtypes of tumors based on the presence of various mutations and gene expression profiles. A recent study by *Chen F et al.* profiled 1023 NSCLC cases from the Cancer Genome Atlas (TCGA). The NSCLC cases were grouped based on DNA methylation, DNA copy alteration, mRNA expression, miRNA expression, and protein expression, and the authors identified nine molecular-based NSCLC subtypes [11]. They identified three squamous (SQ.1, SQ.2a and SQ.2b) and five adenocarcinoma (AD.1 to AD.5) molecular subtypes. With this detailed classification it was also demonstrated that there is some overlap between these molecular SQ subtypes, which was classified as a mixed molecular subtype that consisted of 32% of squamous cell carcinomas. This study demonstrated the wide heterogeneity of these tumors as well as the importance for the better characterization of the molecular subgroups. The five molecular subtypes identified for lung adenocarcinomas was more varied and determined based on differences between CpG

island methylator phenotype, cancer-testis gene expression, immune cell infiltration, immune checkpoint pathway activation, neoepitope count and mutation rate, and p38 and mTOR pathway activation [11]. Furthermore, the Cancer Genome Atlas Research Network has proposed an updated nomenclature for the coordinate naming of the transcriptional subtypes with the histopathological, anatomic and mutational classifications of lung adenocarcinoma. These are the terminal respiratory unit (TRU), the proximal inflammatory (PI), and the proximal-proliferative (PP) transcriptional subtypes [12]. The PP subtype was enriched for *KRAS* mutants, the PI subtype had solid histopathology and co-mutations in *NF1* and *TP53,* and *TRU* consisted of the majority of the EGFR mutated and kinase fusion expressing tumors. These subtypes also displayed different mutation rates, transition frequencies, genomic ploidy profiles, patterns of large-scale aberration, and they differed in their association with smoking history [12]. These molecular subtypes may provide useful information in the clinical setting as they could potentially help predict prognosis, predict therapeutic responses, and specific targets for therapy [11].

The profiling of these molecular subtypes has provided significant insight into these tumors and the potential actionable mutations that can be exploited for the treatment of NSCLC. Commonly mutated genes include *TP53* (46%), *KRAS* (33%), *EGFR* (13% and are mutually exclusive with *KRAS* mutations), and *BRAF* (10%) [12]. Dual mutations are also common in NSCLC, with the most frequent being for *TP53/KRAS* reported at 28-38% [13, 14]. In a study by Jamal-Hanjani *et al*. which investigated the clonal and sub-clonal driver mutations and timing of genomic events in lung adeno- and squamous carcinomas, they determined that mutations in *TP53* were predominantly clonal and occur relatively early in the initiation of both cancer subtypes [15].

Mutations in *KRAS* occurred predominantly late in lung squamous carcinoma, but were found to be an early mutation in lung adenocarcinoma [15]. Identifying targetable mutations has indeed led to the discovery of targeted therapies to treat NSCLC patients. The most common targetable mutations in NSCLC tumours include epidermal growth factor receptor (EGFR) activating mutations, as well as, anaplastic lymphoma kinase (ALK) and ROS proto-oncogene 1 (ROS1) rearrangements. Activating mutations or overexpression of the EGFR and ALK or ROS1 rearrangements have been demonstrated to drive lung cancer formation, as they play an important role in driving tumor growth [16, 17]. Overexpression or activating mutations in EGFR have been identified to be a predictive marker for response to targeted therapies using EGFR tyrosine kinase inhibitors (TKI) in lung cancer [18]. The most common EGFR activating mutations are the LREA amino acid deletion of exon 19 (L747-A750del) and the L858R missense mutation [19]. These mutations account for approximately 85% of EGFR mutations in NSCLC [19]. The use of TKIs, such as erlotinib, gefitinib, and afatanib, have been demonstrated to effectively target these EGFR mutations. EGFR TKIs act as competitive inhibitors of ATP for the tyrosine kinase domain of EGFR thus inhibiting activation of the downstream pathways [20]. It has been demonstrated that the presence of an activating mutation in EGFR can predict response to EGFR TKI [19]. For example, the First-SIGNAL study demonstrated that in never smokers with lung adenocarcinoma that an activating EGFR mutation predicted better overall response rate and progression free survival to gefitinib as a first line therapy compared to chemotherapy [21]. Resistance to erlotinib and gefitinib has been observed in patients due to the emergence of the EGFR T790M mutation which prevents efficient TKI binding [19]. Afatinib, a second-generation irreversible inhibitor of EGFR, has been demonstrated to be a potent inhibitor of both wild-type and activating mutant EGFR. It is FDA-

approved for the first line treatment of metastatic NSCLC with exon 19 deletions or EGFR L858R mutations [22]. It has also been approved in Europe for the treatment of metastatic NSCLC with EGFR T790M mutations [23]. As a first line therapy in NSCLC a significant improvement in progression free survival was observed in patients treated with afatinib compared to gefitinib (LUX-Lung 7) [24]. Significant improvements in progression free survival were also observed in afatinib treated advanced lung squamous cell carcinoma patients compared to erlotinib as a second line treatment (LUX-Lung 8) [25].

Similarly, ALK and ROS1 rearrangements can also be targeted using TKI [6]. It has been demonstrated that due to very similar homology of the tyrosine kinase domains between ALK and ROS1 that both reciprocal rearrangements can be targeted with the same set of TKIs, such as crizotinib [26]. Numerous preclinical and clinical studies have demonstrated improved treatment response and gains in survival for patients treated with these targeted therapies over conventional chemotherapy [27-29]. Unfortunately, only a small subset of patients present with these mutations and while initially responsive to these therapies, many will develop resistance to TKI treatment within 9 to 12 months [6, 18] and eventually will be treated with platinum based chemotherapy regimens. Therefore, the use of DNA-damaging chemotherapies, such as cisplatin, remains a widely employed therapeutic option for the treatment of lung cancer. Additionally, the majority of patients with NSCLC present with advanced disease where surgical resection is not a viable curative treatment option [30]. In such cases, the standard of care involves platinum-based doublet chemotherapy [31].

1.2 Breast Cancer

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among Canadian women [1]. Approximately 26,000 women were diagnosed with breast cancer in 2017 which accounts for 25% of all cancer diagnoses in Canadian women [1]. Five-year overall survival for breast cancer patients is approximately 87% [1] but drops drastically to approximately 20% in patients with advanced metastatic disease [32]. Breast cancer is a very heterogeneous disease and as such characterized into a variety of histopathological and molecular subtypes. The histopathological subtypes of breast cancer are broadly classified as invasive or *in situ* (non-invasive) carcinoma. *In situ* carcinoma is separated in to ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), with DCIS being the more common of the two [33]. Invasive carcinomas are also a very heterogeneous group of tumors. These subtypes include infiltrating ductal (IDC), invasive lobular (ILC), ductal/lobular, mucinous, tubular, medullary and papillary carcinomas. Of the invasive subtypes, IDC is the most common and accounts for approximately 70-80% of invasive lesions [33, 34]. These classifications are further segregated based on the molecular characteristics of the tumors based on the expression of various markers, as well as, the TNM staging which accounts for tumor grade, size, and spread. The molecular markers include the clinically relevant estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2). In addition to these markers, more recent advances have led to the use of molecular profiling based on the expression of multiple markers and gene signatures. *Sorlie et al* were the first to propose and examine the intrinsic subtypes of breast tumors employing an intrinsic gene set of 456

cDNA clones in order to determine and classify the potential molecular subtypes of 78 breast cancer tumors [35]. This study identified five molecular subtypes of breast cancer that were separated in to two branches. The first branch was characterized by low to absent expression of ER and associated transcription factors; the basal-like gene expression pattern, *ERBB2*$^+$ (HER2$^+$) tumours, and normal breast-like groups [35]. The second branch characterized the ER$^+$ groups consisting of the luminal subtypes A, and B + and C. Following this initial classification of molecular subtypes, Sotiriou *et al.* identified six molecular subtypes using 706 probe elements selected for high variability across all tumors in 99 patients [36]. The classification of the molecular subtypes identified by Sotiriou *et al.* overlapped well with those described by Sorlie *et al.* with the addition of a sixth subtype which separated the luminal B+C subtype described by Sorlie *et al.* into luminal-like 2 and 3 [36]. From these initial classifications, many subsequent studies have further characterized the intrinsic and molecular subtypes of breast tumors. More recently, the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) analysis identified 10 intrinsic subtypes of breast cancer based on the stratification of genomic copy number drivers by combining gene expression and DNA copy number alterations [37]. To date, the intrinsic subtypes identified by the METABRIC analysis represent the most extensive molecular-based taxonomy of breast cancer [38]. They determined that six of the identified intrinsic subtypes consisted mostly of ER$^+$ samples and were classified as luminal A or B by the Prosignia Prediction Analysis of Microarray 50 (PAM50) test, while the remaining identified subtypes were either mixed, HER2$^+$, or basal-like [37, 38]. PAM50 tests tumor samples for a cohort of 50 genes and helps both predict the probability of metastasis as well as determine molecular subtype. Similar to previous reports, the METABRIC analysis demonstrated wide

variability in the tumor subtypes and demonstrated the overlap of the various molecular subtypes of breast cancer.

While these studies have demonstrated that the classification of breast cancer into distinct subtypes may be over simplified based on the wide heterogeneity observed, it is still common practice to classify tumors as one of 4 main intrinsic molecular subtypes [39]. The four main molecular subtypes that have been classified are luminal A, luminal B, Her2-enriched, and basal-like [39]. Luminal A tumors are the most common subtype representing 50-60% of all breast cancers [40]. These tumors are typically of a low histological grade and are associated with a favourable prognosis. Luminal A tumors are ER^+ and/or PR^+ and $HER2^-$ with low Ki67 staining (low proliferation index) [41]. Luminal B tumors account for approximately 15-20% of all breast cancers and differ from luminal A tumors as they are more aggressive with a higher histological grade and proliferative index, as well as a poor prognosis [42]. Luminal B tumors are ER^+ and can be either positive or negative for the expression of PR and Her2.. Luminal A and B tumors are largely characterized at the RNA and protein level by the expression of proliferation/cell cycle-related and luminal/hormone-regulated pathways [39]. Luminal A tumors have a lower number of mutations across the genome and less chromosomal copy number changes, as well as, lower rates of *TP53* mutations when compared to luminal B tumors [43]. The Her2-enriched subtype accounts for 15-20% of breast cancers [40]. These tumors are typically more aggressive due to the high expression of the Her2 or Her2 related genes resulting in increased proliferation, invasion and metastasis [40, 44]. At the RNA and protein level, these tumors are characterized by the high expression of Her2-related and proliferation-related genes, intermediate expression of luminal-related genes, and low expression of basal-like genes [39].

Her2-enriched tumors display the highest number of mutations across the genome and 72% possess *TP53* mutations [39]. Lastly, the triple-negative breast cancer (TNBC) subtype of tumorsdo not express ER, PR or Her2. TNBC tumors generally have a high histological grade and high mitotic and proliferative index [40]. Of triple-negative tumors, the basal-like subtype is the most prevalent accounting for approximately 70-80% of TNBC [45]. The basal-like subtype is identified through gene expression profiling, whereas the TNBC refers to the immunohistochemical classification of these tumors identifying them as ER^- PR^- $Her2^-$. Basal-like tumors are an aggressive subtype with poor prognosis due to high rates of metastasis [46]. At the RNA and protein level basal- like tumors are characterized by high expression of proliferation-related genes and keratins which are typically expressed in the basal layer of the skin [39]. Basal-like tumors display the second highest number of mutations across the genome, and 80% possess *TP53* mutations. Mutations in BRCA1, a common hereditary mutation that affects tumour DNA repair have also been associated with triple-negative tumors with the basal-like subtype accounting for approximately 75% of *BRCA1* gene related breast cancers [45]. Claudin-low is another subtype of TNBC that is characterized by low expression of genes involved in tight junctions and cell-cell adhesions. Similar to other TNBC, patients with claudin-low tumors also demonstrate poor clinical outcomes [39].

Treatment of breast cancer is multimodal and can vary depending on many factors. In most cases if the primary tumor is localized and without lymph node involvement surgical resection is performed [47]. In patients with larger tumors or tumors that display nodal involvement neoadjuvant and/or adjuvant chemotherapy, or radiation therapy can be administered [47]. The improved identification and classification of the histopathological subtypes of breast cancer

tumors has led to the improved treatment of breast cancer patients, and has allowed more targeted therapies to be employed. Treatment of ER^+ breast cancers, identified through IHC, typically involves the use of hormone blockade with drugs such as tamoxifen which target the estrogen receptor and its downstream cellular pathways in these tumors as a first –line treatment [47]. Tamoxifen is a selective ER modulator that inhibits tumor growth by preventing estrogen binding to the ER and blocking the proliferative effect of estrogen on mammary epithelial cells [48]. . It is primarily employed in premenopausal women but can also be used in the treatment of postmenopausal women who cannot tolerate aromatase inhibitors. The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis found that adjuvant tamoxifen for 5-years has a proportional reduction on the annual breast cancer mortality rate of 31% for patients with ER^+ breast cancers regardless of patient age [49]. In postmenopausal women, aromatase inhibitors have been demonstrated to improve survival compared to tamoxifen [50]. Aromatase inhibitors prevent the conversion of androgens to estrogen. Currently employed aromatase inhibitors include letrozole, anastrozole, and exemestane. Another therapeutic strategy employed for the treatment of ER^+ breast cancers is the use of ER down-regulators, such as fulvestrant, in postmenopausal women. Fulvestrant prevents ER dimerization and DNA-binding, as well as, increasing ER turnover and degradation which prevent ER signaling [51]. Fulvestrant has been recently demonstrated to improve patient outcome compared to anastrozole treatment in endocrine therapy–naïve postmenopausal women with locally advanced or metastatic ER^+ breast cancer [52].

For the treatment of Her2 positive breast cancers, targeted therapies against the Her2 receptor have demonstrated significant clinical benefit to patients [53] and is the recommended treatment

option for patients that have confirmed amplification of Her2 [47]. The current standard therapy for the treatment of early stage Her2-positive breast cancers consists of neoadjuvant treatment with a combination of chemotherapy and anti-HER2 targeted therapy [54]. The introduction of Her2 targeted therapies has significantly improved patient survival with an increase from approximately 20 months to almost 5 years [55]. Commonly employed Her2 targeting therapeutics include trastuzumab, lapatinib, and ado-trastuzumab emtansine (T-DM1). Trastuzumab is a monoclonal antibody that targets Her2 to prevent its signaling and down-regulates Her2 expression [56]. Initially approved for the treatment of metastatic breast cancer, trastuzumab has demonstrated efficacy in early breast cancers and has become the standard of care for the treatment of Her2-positive breast cancers [56]. Lapatinib is a reversible tyrosine kinase inhibitor that targets both EGFR and Her2 and supresses their downstream signaling [57]. Lapatinib is commonly employed in combination treatments and is usually employed as a third line or beyond treatment option [58] and has been demonstrated to enhance the apoptotic effect of Her2-targeting antibodies [59]. T-DM1 is an antibody-drug conjugate consisting of the microtubule targeting drug emtansine (DM1) linked to trastuzumab. T-DM1 is most commonly employed as a second line therapeutic option following a trastuzumab and chemotherapy regimen [58]. T-DM1 was demonstrated to significantly improve overall survival in breast cancer patients who progressed on two or more HER2-directed regimens compared to the treatment of physician's choice [60].

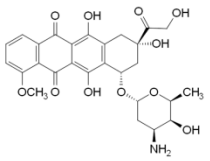
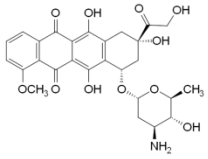
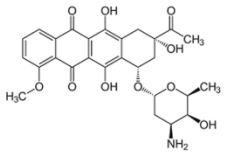
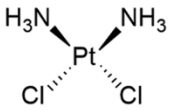
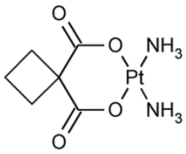
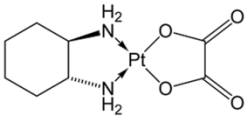
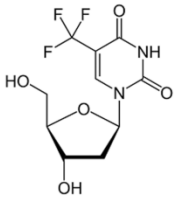
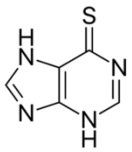
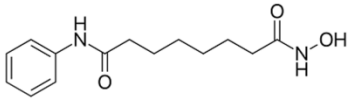
Treatment options for TNBC tumors are limited and heavily rely on the use of chemotherapy, and such tumors have shown sensitivity to this approach [61]. Luminal B and Her2 positive tumors also have been demonstrated to be sensitive to chemotherapy treatment [61, 62]. TNBC

tumors display the most complete response (~22%) to chemotherapy treatment among breast cancer subtypes [55], but they also have higher rates of recurrence and metastasis compared to the other subtypes [63]. The lack of expression of hormone receptors or Her2 prevents the use of targeted therapies that are currently employed for the treatment of other breast cancer subtypes. Therefore, chemotherapies, such as platins, anthracyclines, and taxanes, are widely employed for the treatment of these tumors, and they can be employed in combination with bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF) [55]. Recent work has shown that 9- 100% (mean 35%) of triple negative breast cancers are mutated for *BRCA1* and it was additionally determined that up to 66% of *BRCA1* tumors are triple-negative [64]. This mutation has been identified to sensitize tumor cell to the use of PARP inhibitors, such as olaparib, as well as carboplatin [55]. *BRCA1* and *BRCA2* play an important role in the homologous recombination DNA-repair pathway as well as DNA-replication fork stabilization [65]. It has been reported that germline mutations in these genes have a positive effect on treatment response with carboplatin but not on taxanes which are the standard of care for advanced breast cancers [66, 67]. The presence of *BRCA1* and *BRCA2* mutations has also lead to the use of PARP inhibitors as single agents in these tumors as they are able to exploit the synthetic lethality of loss of BRCA1/2 and PARP [66, 67].

While targeted therapies have proven to be effective for the treatment of breast cancer, in many cases resistance to these treatments occurs. In such cases, conventional cytotoxic chemotherapies are required for their treatment. Commonly employed chemotherapies for the treatment of breast cancer include anthracyclines (doxorubicin and epirubicin), platins (cisplatin and carboplatin), nucleoside analogues (5-fluorouracil and capecitibine), and taxanes (docotaxel

and paclitaxel). Anthracycline based treatments, typically the combination of doxorubicin and taxol, have become the standard of care for breast cancer patients receiving chemotherapy [47] with approximately 32% of all breast cancer patients receiving anthracycline-based chemotherapy [68].

Figure 1: Structure of common DNA-damaging chemotherapies of the various classes of DNA-damaging agents presented in this thesis. Additionally, the structure of the HDAC inhibitor Vorinostat is presented as it is relevant to this thesis.

	Doxorubicin	Epirubicin	Daunorubcin
Anthracyclines			
	Cisplatin	Carboplatin	Oxaliplatin
Platins			
	Trifluridine	6-Mercaptopurine	
Nucleoside Analogues			
	Vorinostat		
HDAC Inhibitror			

1.3 DNA-Damaging Chemotherapeutics

DNA-damaging chemotherapeutics have been one of the most successful and widely employed classes of drugs for the treatment of cancer. Treatment with DNA-damaging agents results in the accumulation of lethal levels of DNA-damage which if tumor cells are unable to overcome leads to tumor cell death. Various classes of DNA-damaging drugs have been identified and employed in the treatment of cancer. The most common agents employed in breast cancer patients are the topoisomerase inhibitors (such as anthracyclines), alkylating agents (such as platins), and nucleoside analogues which all have distinct mechanisms for the induction of DNA-damage (Figure 1).

1.3.1 Platins

Platinum-based chemotherapies are one of the most widely employed class of drugs for the treatment of cancer. It is estimated that approximately half of all patients undergoing chemotherapy treatment receive platinum-based drugs [69]. Following the initial discovery of the anticancer activity of cisplatin, a large number of platinum-based drugs have been synthesized and tested, of which three have emerged as the main platins for the treatment of a number of cancers, including breast, ovary, lung, head and neck, and cervical cancers [70]. These three platinum-based drugs are cisplatin, carboplatin, and oxaliplatin (Figure 1). Platins are mainly considered DNA-damaging agents and typically form bifunctional DNA crosslinks. By performing structure activity relationship studies it was determined that for platins to have activity they require a square-planar geometry, to be charge neutral, contain two cis-ammine

ligands, and have two cis-anionic ligands[69]. Platins exert their cytotoxic effect through four major steps, i) cellular uptake, ii) aquation/activation, iii) DNA platination, and iv) cellular processing of Pt–DNA lesions [71].

Cisplatin was the first platinum-based drug to be approved for the treatment of cancer and has remained a mainstay in current chemotherapeutic regimens. Cisplatin has been demonstrated to be highly effective in the treatment of testicular cancer, where its use can achieve up to a 90% cure rate [72]. It is also a highly active agent in the treatment of NSCLC, breast, and ovarian cancers. The main toxicities associated with cisplatin use are ototoxicity, nephrotoxicity and neurotoxicity, which can hinder its use [70]. Carboplatin was identified as an agent that displayed similar effectiveness as cisplatin, but demonstrated less toxicity [72]. Carboplatin differs in structure from cisplatin as it contains a bidentate dicarboxylate (CBDCA) ligand in place of the two chloride ligands that acts as the leaving group [73]. This makes carboplatin less reactive than cisplatin with slower DNA-binding kinetics [73]. Carboplatin induces the same DNA-adducts as cisplatin but needs to be administered at approximately a 10 times higher dose, and it requires a longer incubation time [70]. Carboplatin treatment has a significantly reduced risk of nephrotoxicity, but is associated with myelosuppression which can be dose limiting [70, 73]. Carboplatin is mainly used in the treatment of ovarian, head and neck, and small-cell lung cancers. Oxaliplatin is a third generation platinum-based chemotherapy. It differs from cisplatin as it comprised of a platinum atom complexed with oxalate and diaminocyclohexane (DACH). The larger DACH is believed to contribute to the increased cytotoxicity of oxaliplatin and confer a lack of cross-resistance of oxaliplatin compared to cisplatin and carboplatin [72]. Oxaliplatin was the first platin to demonstrate convincing clinical efficacy in metastatic colorectal cancer,

but it is more effective as part of combination therapies [72]. The dose-limiting toxicity of oxaliplatin treatment is sensory neuropathy [70].

Acquired or intrinsic resistance to platins, as well as, the associated toxicities of treatment have lead for the need of predictive biomarkers of platin response, in order to avoid exposing patients to toxicities without receiving clinical benefit and in order to improve patient outcome and drive the use of other more potentially efficacious therapies [74]. Currently, the leading biomarkers for predicting response to platinum based therapy are the expression of proteins involved in DNA repair. Sensitivity to platins is inversely proportional to the ability of the cell to repair DNA adducts [74]. ERCC1 is a key component of the nucleotide excision repair pathway which is responsible for repairing the platinum-DNA adducts formed following treatment. Multiple studies have demonstrated the potential predictive capacity of ERCC1 for the treatment of NSCLC with platins. Zhou *et al* identified a polymorphism in *ERCC1* (C8092A) which alters ERCC1 mRNA expression and stability as a potential predictor of platin sensitivity, as there was a significant association between the polymorphism and overall survival in NSCLC patients [75]. Other studies have investigated the mRNA expression levels of ERCC1 and have demonstrated that increased ERCC1 expression is associated with platin resistance in NSCLC, ovarian, and gastric cancers [76-78]. Unfortunately, there have been some challenges in utilizing ERCC1 as a predictor of response due to large variation across ERCC1 evaluation procedures [79]. However, ERCC1 remains to be a highly promising biomarker for the prediction of platin response and the development of a relevant standardized assays may allow for its clinical utility.

Throughout this thesis, cisplatin was chosen as the main platin of focus and it is further discussed in the following sections.

1.3.2 Cisplatin

Cisplatin (cis-Diaminedichloroplatinum(II)) is a DNA-alkylating agent used in the treatment of many solid tumors, particularly lung, head and neck, and ovarian carcinomas [80]. Cisplatin's cytotoxic effect occurs through its ability to induce DNA-damage through the formation of DNA-adducts [80]. Cisplatin is capable of diffusing through the cell membrane where it can readily interact with DNA. This targeting of the DNA results due to the positive charge of the platinum ion which favours interaction with the negatively charged nucleic acids of DNA [81]. To form DNA-adducts, cisplatin is activated through the hydrolysis of its chlorine leaving groups which allows for its covalent binding to DNA [82]. Cisplatin preferentially forms DNA-adducts with guanine residues and bi-functional DNA- adducts are formed when both chlorines of cisplatin are removed allowing for crosslinking of two adjacent guanines on the same DNA-strand or on separate strands [80]. The accumulation of the DNA-adducts is considered the initiating step in cisplatin's anti-cancer effect that is followed by cell cycle arrest and inhibition of DNA-replication [80]. When the DNA-damage burden within the cell cannot be repaired, apoptotic pathways are initiated resulting in tumor cell death. Cisplatin has also been demonstrated to initiate cell death through the activation of the endoplasmic reticulum stress pathway [83]. *Mandic A et al* demonstrated that the treatment of enucleated cells with cisplatin induced cell death through the activation of an endoplasmic reticulum specific caspase [83].

Cisplatin can also target mitochondrial DNA, and it was demonstrated that cisplatin adducts can accumulate 300-500 times more in the mitochondria than in nuclear DNA [84].

1.3.3 Cisplatin use in Non-Small Cell Lung Cancer (NSCLC)

Cisplatin is one of the most active agents employed for the treatment of NSCLC [80]. Following numerous clinical evaluations, cisplatin combination therapy demonstrated enhanced efficacy in comparison to a wide range of other chemotherapeutic regimens and is now considered first-line therapy in the advanced (metastatic) setting [85]. First line therapeutic regimens typically consist of platin-doublet chemotherapy (i.e. cisplatin with taxol). However, overall gains in survival have been modest with combination therapy, with a median survival of 12 months in patients who receive platin-doublet chemotherapy compared to 4 months in untreated patients [86]. Response rates to treatment are approximately 30% and have not been demonstrated to be durable in patients, which results in a modest effect on overall patient survival [87]. Clearly, novel therapeutic strategies are urgently required.

1.3.4 Cisplatin Resistance

Although cisplatin, and other platinum-based agents, are effective in the treatment of cancer, resistance to these drugs is often observed and is an impediment to their efficacy [88-91]. Most common mechanisms of resistance involve DNA-damage response (DDR) factors [92]. Inter- and intrastrand crosslinks formed by cisplatin are mainly repaired through the nucleotide excision repair (NER) pathway. NER is initiated through the sensing of DNA-adducts and

helical-distortion by a protein complex containing XPC [93]. XPC leads to the recruitment of the NER machinery including the transcription initiation factor IIH complex, for DNA-damage verification, and XPF-ERCC1, which excises the DNA-adduct [92]. Loss of NER increases the sensitivity of cells to cisplatin treatment [92]. Excision-repair cross complementation group 1 (ERCC1) expression has been correlated with cisplatin treatment outcome, with decreased expression correlated with enhanced progression free survival (PFS) and overall survival (OS) [94]. In ovarian cancer patients, increased mRNA and protein expression of ERCC1 have also been correlated with cisplatin resistance [80, 94].

Cisplatin resistance can also result due to mechanisms involved in its influx and efflux, or cellular sequestration. Cisplatin's entry into the cell can occur in two ways i) passive diffusion through the cell membrane, or ii) through membrane transport proteins [80]. Copper transporter-1 (CTR1) is a membrane transport protein that has been implicated in cisplatin uptake since its loss results in cisplatin resistance [95, 96]. The efflux proteins ATPase copper transporting beta (ATP7B) and multidrug resistance protein 2 (MRP2) have also been implicated in cisplatin resistance. Overexpression of ATP7B was demonstrated to sequester cisplatin in vesicles and confer resistance to human epidermoid carcinoma cells [97]. Similarly, knockdown of MRP2 sensitized oral squamous cell carcinoma cells to cisplatin treatment [98]. Cisplatin has a high affinity to bind sulphur and can readily bind to the sulphur containing amino acids of many proteins [80]. Proteins such as glutathione and metallothionein, which are rich in cysteine and methionine, can bind and inactivate cisplatin. High expression levels of these proteins have also been linked to cisplatin resistance in *in vitro* and *in vivo* [80, 82, 99].

1.3.5 Anthracyclines

Anthracyclines were originally isolated from the bacterium *Streptomyces peucetius* [100]. Daunorubicin was the first to be identified and demonstrated significant clinical activity in the treatment of pediatric acute leukemia [100]. Further development lead to the discovery of doxorubicin followed by the development of other anthracyclines including epirubicin, idarubicin. Structurally, all anthracyclines consist of a tetracyclic aglycone (quionone) structure linked to the aminosugar daunosamine (Figure 1) [101]. Small structural differences to the aminosugar or side chain of anthracyclines result in changes to their clinical activity and toxicity. Doxorubicin differs from daunorubicin by the presence of a hydroxyl group on its side chain. Epirubicin is an epimer of doxorubicin and differs structurally due to the orientation of the hydroxyl group on the daunosamine. Anthracyclines have been demonstrated to be active in many cancers, with doxorubicin demonstrating the broadest activity in cancers including lung, ovarian, gastric, thyroid, breast, sarcoma, and Hodgkin's and non-Hodgkin's lymphomas [100]. Epirubicin is used in breast, esophageal and gastric cancers [102]. Anthracyclines are considered topoisomerase inhibitors and are potent inducers of DNA-double strand breaks. Topoisomerases are enzymes responsible for the modification of DNA topology without modifying the deoxynucleotide sequence. Topoisomerases form transient single or double strand breaks in DNA in order to change the twisting status of the DNA-double helix which is altered depending on cell cycle phase and transcriptional activity [103]. Anthracyclines intercalate into DNA and form a complex with topoisomerases and DNA, inhibiting their function, and resulting in DNA breaks.

While the widely accepted mechanism of anthracycline action is through DNA intercalation and topoisomerase inhibition, other mechanisms of cytotoxicity have been identified which can have anti-tumor effects or result in unwanted toxicities. Treatment with anthracyclines, doxorubicin and daunorubicin, is strongly associated with cardiotoxicity which has been attributed to the generation of reactive oxygen species (ROS) and the inhibition of topoisomerase 2β (Top2β). ROS is produced by the quinone moiety of anthracyclines which is reduced by cellular oxido-reductases to form a semiquinone radical [101]. The semiquinone radical is able to react with oxygen to produce the parent anthracycline and a superoxide anion, and this reaction allows for a continuous redox cycle which results in superoxide anions accumulating within the cell [101, 104]. Top2β expression is more abundant in quiescent cells, such as cardiomyocytes, and its inhibition by anthracyclines leads to disruption of various signaling pathways, such as p53 signaling, mitochondrial dysfunction, and increased apoptosis [101]. Loss of Top2β expression has been demonstrated to protect cardiomyocytes from doxorubicin treatment in mice [105]. These mechanisms may also contribute to the tumor cell cytotoxicity of anthracyclines. *Mai Y et al*. demonstrated that in activated B-cell diffuse large B-cell lymphomas that doxorubicin induced oxidative stress often plays an important role in tumor cell cytotoxicity [106]. ROS has also been demonstrated to play a role as signaling molecule for doxorubicin induced cell death in osteosarcoma [107]. Similarly, in human leukemia cell lines it was demonstrated that the oxidative DNA-damage induced by doxorubicin treatment was a main initiator to cellular apoptosis [108]. Other studies have demonstrated that the use of anti-oxidants or ROS scavengers can attenuate the tumor-cell cytotoxicity of doxorubicin. *Lee Y et al*. demonstrated that treatment with n-acetylcysteine (NAC) reduced doxorubicin induced apoptosis in *TP53* wild-type tumor cell lines, but interestingly demonstrated that NAC synergistically enhanced

doxorubicin induced apoptosis in *TP53* altered cells [109]. This suggests that ROS can play an important role in mediating anthracycline tumor cell cytotoxicity as many tumors have alterations in *TP53*. Other mechanisms of anthracycline induced tumor cell cytotoxicity include ceramide production, and histone eviction [110]. Doxorubicin was demonstrated to upregulate ceramide production in MCF7 breast cancer cells [110]. It was also found that upregulation of glucosylceramide synthase, which converts ceramide to glucosylceramide, is associated with doxorubicin resistance [111]. Additionally, the stimulation of ceramide by doxorubicin treatment was found to upregulate the transcription factor, CREB3L1, which results in reduced proliferation due to the upregulation of genes involved in cell cycle arrest [112]. Overexpression of CREB3L1 was shown to enhance the sensitivity of MCF7 cells to doxorubicin [112]. Lastly, anthracyclines have been demonstrated to evict histones. *Pang B et al*. demonstrated that doxorubicin and daunorubicin can evict histones from open chromatin which results in decreased DNA repair and is an important mediator of apoptosis in acute myeloid leukemia blasts [113].

Epirubicin was developed in order to limit the cardiotoxicity that is observed with doxorubicin treatment. The positional change of the hydroxyl group on daunosamine from axial to equatorial had little effect on the activity and mechanism of action of epirubicin as compared to doxorubicin [103]. Treatment with epirubicin has been demonstrated to be less cardiotoxic than with doxorubicin. A meta-analysis of 55 randomised controlled trials, mostly consisting of women with advanced breast cancer, determined that there is a reduced risk of cardiotoxicity in patients treated with epirubicin compared to doxorubicin [114]. No difference was observed in the anti-tumour efficacy between epirubicin and doxorubicin [114]. Additionally, the structural change in epirubicin contributes to pharmacokinetic and metabolic changes such as increased

volume of distribution, 4-*O*-glucuronidation, and shorter terminal half-life [103]. This permits higher doses of epirubicin to be employed. The median dose associated with cardiotoxicity for doxorubicin is 468mg/m^2 compared to 935mg/m^2 for epirubicin [100].

Due to the toxicities associated with anthracycline use, there has been an effort to identify predictors of response to determine which patients will benefit from anthracycline based chemotherapeutic regimens. In breast cancer, where doxorubicin and epirubicin are widely employed, analysis of 3,846 patient samples determined that patients with duplication of the chromosome 17 pericentromeric alpha satellite (CEP17) or aberrations in *TOP2A* benefit from anthracycline based chemotherapeutic regimens [115]. Chromosome 17 contains many genes that are important in breast cancer and DNA-repair, including *HER2*, *TOP2A*, *BRCA1*, and *TP53* [116]. This chromosome also contains regions that display significant genomic instability, such as the 17q12-q21 chromosomal region that incorporates *HER2* [116]. Chromosomal instability is a frequent event in cancers and results in chromosomal aberrations. Cell cycle checkpoints play an important role in ensuring proper cell division and chromosomal segregation, but when aberrations occur in proteins involved in these processes, improper checkpoint regulation can lead to chromosomal instability as well as missegregation of chromosomes. Previous reports have demonstrated that an intact spindle assembly checkpoint is required for mitotic cell death induced by anthracyclines [116]. Work by *Carter S et al*. has identified a gene signature that can predict chromosomal instability referred to as CIN25 [117]. This signature contains genes that are involved in DNA replication, chromosome segregation and cell cycle checkpoint control. Further work has demonstrated that this signature can be used to predict the efficacy of anthracycline treatment in breast cancer patients [118]. *Spears M et al*. further demonstrated that

the independent predictor signature of four genes, the CIN4 which consists of genes involved in DNA-repair/DNA-binding activity, could also be used to predict patient benefit from anthracycline based treatments [118]. These works have demonstrated the importance of the dysregulation DNA-repair genes and the dysregulation of mitotic checkpoints in predicting anthracycline sensitivity. It also suggests that patients who will receive clinical benefit from anthracycline treatment are likely to have dysregulated mitotic checkpoints and indicates a key mechanism of action for anthracyclines cytotoxicity. It also provides a potentially clinically useful signature for predicting patients who will benefit from anthracycline treatment.

1.3.6 Doxorubicin

Doxorubicin, also known as Adriamycin, belongs to this class of anthracycline drugs and will be a focus of this study. Doxorubicin has demonstrated significant efficacy for the treatment of solid tumors, such as breast, cervical, lung, stomach, and ovarian [119]. It is also widely used for the treatment of hematological malignancies [119]. Primarily considered to be a DNA-damaging agent, doxorubicin's mechanism of action has been identified to be through the targeting of topoisomerase IIα (TopIIa) [120]. Topoisomerases are a class of proteins involved in the unwinding/untangling of DNA strands. Topoisomerase inhibitors prevent the rearrangement of DNA strands resulting in stalled replication [120]. Additionally, they induce DNA-damage through the stabilization of topoisomerase-DNA complexes. Doxorubicin targets topoisomerases in two ways: i) it intercalates DNA which prevents topoisomerase binding and results in stalled replication forks, and ii) it stabilizes topoisomerase IIα resulting in the formation of double strand breaks (DSB) (Figure 2) [120]. Both of these mechanisms result in the formation of the

double strand breaks that elicit a DDR. Other actions of doxorubicin have also been identified which add to its cytotoxic properties, including the formation of DNA adducts and free radicals that can further damage DNA as well as cellular proteins [110].

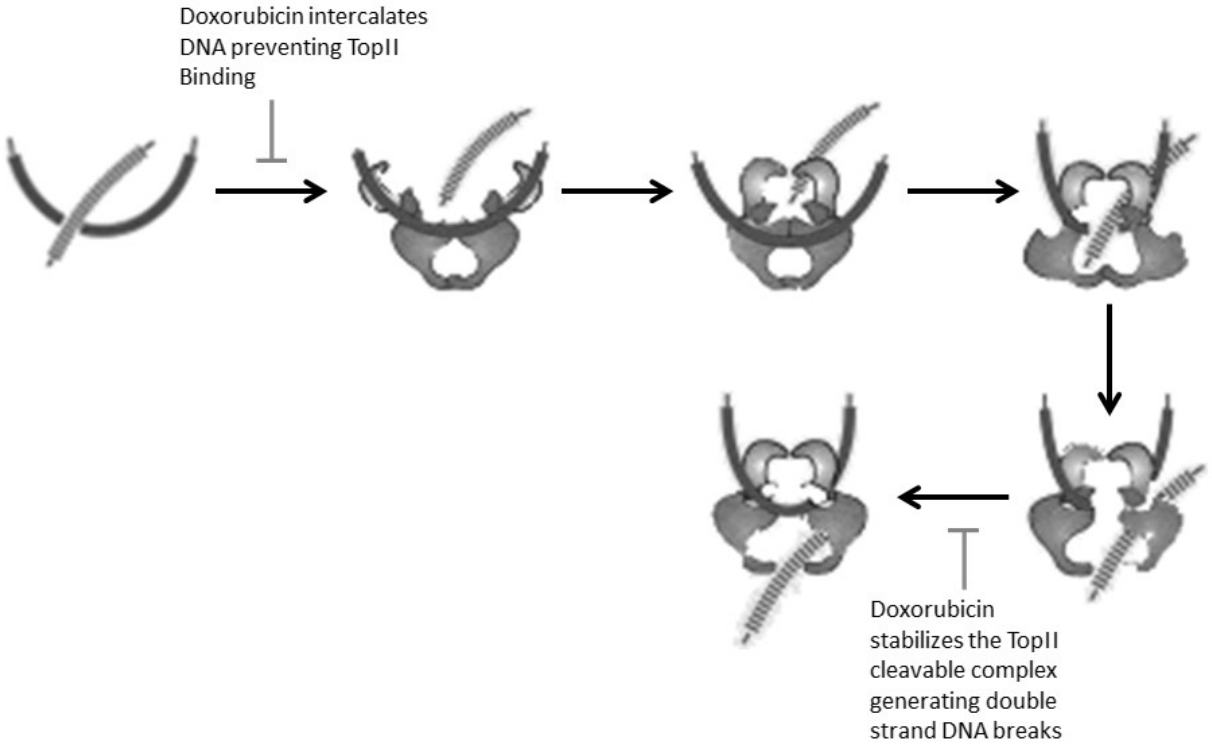
1.3.7 Doxorubicin Use in Breast Cancer

Doxorubicin is widely employed in the treatment of breast cancer [121] as the majority of breast cancer patients receive doxorubicin either as a neoadjuvant (prior to primary treatment) and/or as part of a primary treatment regimens [122]. Doxorubicin has been demonstrated to be effective in the treatment of early breast cancers when employed in combination regimens [123]. The EBCTCG demonstrated that anthracycline containing adjuvant chemotherapy reduces the 10-year relative risk of recurrence from 47.4% to 39.4% [123]. Additionally, the EBCTCG analysis demonstrated a decrease in recurrence and mortality when taxane was added to anthracycline treatment regimen [123]. With these findings, anthracycline based chemotherapy regimens have become the standard for adjuvant treatment of early breast cancer [124]. In the advanced breast cancer setting, doxorubicin is typically employed in combination with docetaxel as a first line chemotherapeutic regimen [125]. This combination has demonstrated improved time to progression and overall response rates compared to other combination strategies such as, doxorubicin/ cyclophosphamide, or doxorubicin/ fluorouracil/ cyclophosphamide [126, 127]. Unfortunately, there are significant dose limiting side effects associated with doxorubicin treatment, with the most severe being cardiotoxicity [119]. These associated toxicities result in having to limit the dose patients receive, which ultimately results in decreased effectiveness

[119]. Additionally, doxorubicin's use is also limited due to acquired resistance of tumor cells to this drug.

More recently there has been a shift towards identifying treatments that will reduce disease recurrence without severe toxicity and this has led to further investigation in to anthracycline-free chemotherapy regimens. It was demonstrated that in early breast cancer patients a treatment regimen of docetaxel/cyclophosphamide had a greater overall survival benefit compared to doxorubicin/cyclophosphamide treatment [128]. Additionally, one caveat with early trials investigating the efficacy of anthracyclines was the inclusion of patients with Her2 positive disease which has been demonstrated to increase sensitivity to anthracyclines and may have resulted in an overestimation of the clinical benefit of anthracyclines [129]. This was evaluated by the ABC trials which ultimately found that anthracycline based chemotherapeutic regimens should be employed in breast cancer patients with a high risk of recurrence, such as triple-negative or Her2 negative disease [129, 130]. Doxorubicin still remains a widely used chemotherapeutic for the treatment of breast cancer but with the development of predictive tools it is now possible to employ doxorubicin in more strategic manners to improve clinical benefit and reduce toxicities.

Figure 2: Doxorubicin targets topoisomerase IIα. By intercalating in to DNA, doxorubicin prevents topoisomerase binding and which results in the inability of topoisomerase to untangle the DNA strands leading to stalled DNA replication. Secondly, doxorubicin stabilizes the TopII cleavable complex after it has formed a double strand break, preventing the relegation of the DNA strand. Adapted with modifications from Pommier Y et al. *Chem Biol.* 2010 [120]


Doxorubicin intercalates
DNA preventing TopII
Binding
Doxorubicin
stabilizes the TopII
cleavable complex
generating double
strand DNA breaks

1.3.8 Doxorubicin Resistance

The most characterized mechanism of doxorubicin resistance is the role of drug efflux through ATP-binding cassette (ABC) transporter, P-glycoprotein (P-gp) [131]. Interestingly, inhibition of P-gp has not demonstrated clinical benefit for patients treated with doxorubicin, which suggest the importance of other mechanisms of resistance [131]. This may be due to the evidence of elevated P-gp expression in resistance to doxorubicin treatment mostly coming from *in vitro* models where P-gp is overexpressed, or generation of drug-resistant cells through gradual exposure to increasing doses of doxorubicin, both of which are not representative of what occurs in patients. When P-gp expression is evaluated as a predictor of response to doxorubicin treatment there is limited evidence that its expression levels can predict clinical response to doxorubicin [132, 133].

Due to the basic properties of anthracyclines, they can be protonated and sequestered in the lysosome, which prevents their accumulation in the nucleus and subsequent cytotoxicity [134-136]. Lysosomal alkalinisation, which prevents drug accumulation within the lysosomes, can restore drug sensitivity *in vitro* [137]. Another mechanism of doxorubicin resistance involves its target, TopIIa, as decreased levels of TopIIa results in resistance to doxorubicin in *in vitro* and *in vivo* models [138]. Similarly, increased TopIIa expression increases the sensitivity of tumor cells to doxorubicin treatment [139]. Interestingly, it was demonstrated that altered TopIIa expression, characterized by amplification or deletion used in combination as a single variable, could predict clinical benefit to anthracycline treatment, whereas, either amplification or deletion of TopIIa alone did not have a significant relationship with OS and RFS [115]. The contradicting

results of amplification and deletion of TopIIa predicting clinical response to anthracycline treatment may be related to chromosome instability resulting in amplification or deletion of this gene. Therefore, amplification and deletion of TopIIa as a predictor of anthracycline response may represent a surrogate marker of chromosome instability which has previously been implicated in the response to anthracyclines.

Factors that control TopIIa activity have also been linked to doxorubicin resistance. One such factor is the SWI/SNF chromatin remodelling complex that loads TopIIa onto DNA, and has been found to be frequently mutated in cancer [140]. Other mechanisms of doxorubicin resistance include regulators of DNA-repair through homologous recombination and upregulation of detoxifying enzymes. Aldo-keto reductases (AKRs) convert doxorubicin to doxorubinol which reduces its cytotoxicity. Inhibition of AKRs restored doxorubicin accumulation and localization within the nucleus of resistant cells, and increased its cytotoxicity [141].

1.3.9 Nucleoside Analogues

Nucleoside analogs are a class of anti-metabolite drugs that target essential biological processes or are incorporated into DNA and RNA, and inhibit their normal function [142]. These compounds mimic physiological nucleosides and are incorporated in to newly synthesized DNA or RNA, and/or can target enzymes responsible for nucleotide metabolism [143, 144]. The nucleoside analog family consists of various purine and pyrimidine analogs such as thiopurines (e.g. 6-mercaptopurine and 6-thioguanine) and fluoropyrimidines (e.g. 5-fluorouracil,

trifluridine, and capecitibine). Nucleoside analogues have demonstrated activity in the treatment of a variety of cancers, including breast, colorectal, head and neck, gastrointestinal, pancreatic, and lung tumors, as well as, hematological malignancies [145]. Nucleoside analogues share common mechanism characteristics including cell membrane transporter mediated cellular entry, activation intracellularly by metabolic enzymes, formation of active phosphorylated derivatives, and short half-life [145, 146]. Of relevance to this thesis are the family of fluorinated pyrimides, including 5-fluorouracil, capecitbine, and trifluridine.

5-fluorouracil (5-FU) is the most widely employed fluoropyrimidine. It is widely used in the treatment of colorectal, gastric, and breast cancers. 5-FU anticancer activity is dependent on its transformation in to three active metabolites; Fluorodeoxyuridine monophosphate (FdUMP), Fluorodeoxyuridine triphosphate (FdUTP), Fluorouridine triphosphate (FUTP) [144]. FdUMP inhibits thymidylate synthase (TS), which is an enzyme that plays a critical role in DNA synthesis [147]. TS is responsible for mediating the rate-limiting step in pyrimidine production and the formation of deoxythymidine triphosphate (dTTP). Inhibition of TS results in reduced intracellular levels of dTTP and accumulation of deoxyuridine monophosphate (dUMP), which results in uracil and fluorouracil misincorporation in to DNA and RNA, as well as, disrupted DNA synthesis [147]. FdUTP can be directly incorporated in to DNA which results in DNA-damage [144], and FUTP is incorporated in to RNA which inhibits the processing of preribosomal RNA in mature ribosomal RNA [148]. 5-FU is typically administered intravenously since oral administration results in poor absorption due to degradation by dihydropyrimidine dehydrogenase (DPD) [147]. One strategy that has been employed to

improve the administration, efficacy, and safety of 5-FU treatment has been the development of orally administered pro-drugs, such as capecitibine.

Capecitibine, is converted to 5-FU through three enzymatic reactions. It is first converted to 5′-deoxy-5-fluorocytidine (5′-dFCR) in the liver by carboxylesterase, followed by conversion to 5′-deoxy-5-fluorouridine (5′-dFUR) by cytidine deaminase, in the liver or tumor tissue [144]. The conversion of 5'-dFUR to 5-FU occurs in the tumor as is mediated by thymidine phosphorylase [144]. It has been observed that tumors express higher levels of TP, which results in higher concentrations of 5-FU in cancerous cells [144, 149]. Capecitibine treatment results in comparable plasma accumulation of 5-FU as compared to intravenous administration of 5-FU, and it demonstrates a similar efficacy and toxicity profile compared to 5-FU treatment [144]. Additionally, capecitibine has demonstrated synergistic activity with a wide range of other cytotoxic chemotherapies, including taxanes, anthracyclines, and oxaliplatin [150]. Of relevance to this thesis, recent advances have demonstrated that capecitibine is effective in the treatment of metastatic breast cancer [151]. The incorporation of capecitibine into standard chemotherapy or addition of capecitibine to standard chemotherapy was also shown to improve overall survival in early stage breast cancer patients by a recent meta-analysis of randomized controlled trials [151].

1.3.10 Trifluridine

Trifluridine (α, α, α- trifluorothymidine) is a fluorinated thymidine which act as a nucleoside analogue to induce DNA-damage. It has been demonstrated to have two mechanisms of action;

i) its ability to inhibit thymidylate synthase which is required for thymidine nucleotide synthesis and ii) its ability to be incorporated directly into DNA resulting in strand breaks and apoptosis [152]. Trifluridine is currently FDA-approved for the treatment of metastatic colon cancer in combination with a thymidine phosphorylase inhibitor tipiracil [153]. 5-flurouricil, another fluorinated nucleoside analogue, has also been used in the treatment of metastatic colon cancer, as well as in the treatment of breast cancer [152].

Trifluridine use has been very limited due to its poor bioavailability [154]. In humans, when administered intravenously trifluridine has an elimination half- life of only 18 minutes [154]. More recently, it has been demonstrated that the combination of a thymidine phosphorylase inhibitor, tipiracil, can greatly improve the bioavailability of trifluridine [155]. DNA-incorporation is considered the primary anti-cancer mechanism of this combination treatment. Trifluridine is phosphorylated upon administration to its monophosphate which acts as reversible inhibitor of thymidylate synthase [153, 156]. Further phosphorylation to form its triphosphate metabolite allows for trifluridine incorporation in to DNA by polymerase α where it is most commonly paired with adenine [157]. Incorporation of trifluridine in to DNA results in DNA instability [158]. Moreover, trifluridine incorporation in to DNA in tumor tissue is much higher than that of normal tissue, which may be explained by the upregulation of the pyrimidine metabolic pathway in cancer [157]. Mechanisms of resistance to trifluridine have not been well characterized.

1.4 Histone Deacetylase (HDAC) Inhibitors

Histone acetylation and deacetylation are part of a regulatory mechanism that regulates the transcriptional activity of genes. Acetylation of histones, by histone acetyltransferases (HAT), increases the accessibility of the chromatin to transcription machinery and allows for active transcription [159]. Conversely, deacetylation of histone, by HDACs, results in the condensation of chromatin and gene silencing. HDACs are divided in to four classes. Class I (HDAC1, 2, 3, and 8), II (HDAC4-7, 9 and 10), and IV (HDAC11) which are zinc dependent deacetylases [160]. Class III comprises of the sirtuin proteins (SIRT1-7) which have NAD+ dependent activity and are not inhibited by HDAC inhibitors (HDACi) [160]. HDACi are a class of drugs that prevent histone deacetylation and therefore help maintain areas of DNA with active transcription. There are four classes of HDACi which are the short-chain fatty acids, hydroxamic acids, benzamides, and cyclic tetrapeptides [160]. In cancer, it has been established that the balance between HAT and HDAC activity can be disrupted [159]. Overexpression of HDACs has been reported in breast, colon, prostate, and gastric cancers and can result in the silencing of important tumor supressing genes [160]. It is important to note that HDAC activity is not limited to histones and also have other protein targets.

The anti-cancer effect of HDAC inhibition is generally thought to occur through its effect on regulators of cellular pathways involved in cancer development. The regulation of these processes is achieved through the inhibition of histone deacetylation which results in the modulation of gene expression [161-163]. Additionally, targeting proteins involved in gene

expression, differentiation, and cell death have been demonstrated as a mechanisms of HDACi cytotoxicity [164].

Cell death induced by HDACi has been attributed to the multiple mechanisms. The extrinsic apoptotic pathway, which regulates apoptosis through death receptors and caspase activation, has been demonstrated to regulate HDACi cytotoxicity. HDACi was demonstrated to induce cell death through the upregulation of the expression of death receptor 5 (DR5) and first apoptosis signal receptor (Fas), as well as their respective ligands, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and FasL, in a leukemia cell model [165]. Similarly, caspase activation and cell death was observed in neuroblastoma cells treated with HDACi and was associated with increased expression of Fas and FasL [166]. The intrinsic apoptotic pathway has also been implicated in mediating HDACi induced cell death. Xu et al report that HDACi treatment upregulated pro-apoptotic factors (Bak, Bax, Bim, Bmf, and Bik), downregulates pro-survival factors (XIAP and survivin), and inhibits anti-apoptotic factors (Bcl-2) in prostate cancer cells [167]. Other mechanisms of HDACi induced cell death may be through the production of ROS and subsequent DNA-damage [168], and modulation of the expression of cell cycle regulators, such as p21 [169].

Vorinostat was the first HDACi approved for the treatment of cancer, gaining FDA-approval for the treatment of cutaneous T-cell lymphoma [170]. It belongs to the hydroxamic acid class of HDAC inhibitors and inhibits the enzymatic activity of class I (HDAC1, HDAC2, and HDAC3) and class II (HDAC6) HDACs. Inhibition of these HDACs has been demonstrated to induce the transcription of genes associated with growth arrest, differentiation, and apoptosis [171, 172].

The mechanism of vorinostat induced cytotoxicity is not well characterized, but it may be due to the activation of transcription of genes that are involved in mediating its anti-proliferative effect [173]. Studies have demonstrated that 5- 10% of genes have altered expression following vorinostat treatment [174, 175]. Treatments with HDACi have been demonstrated to upregulate p21 expression and subsequently lead to G1 cell cycle arrest [176]. Additionally, many non-histone proteins are also acetylated, such as p53, which regulates their activity [177]. In breast cancer cell lines, treatment with vorinostat results in the accumulation of acetylated heat shock protein 90 (HSP90) [178]. HSP90 is a required chaperone for the stabilization of proteins that are involved in cancer cell proliferation, such as, the human epidermal growth factor receptor (ErbB-1, EGFR, epidermal growth factor receptor 2 (ErbB-2, Her-2), the fusion oncogene Bcr-Abl, and signaling transduction molecules such as Akt [179]. Increased acetylation of HSP90 leads to the degradation of these targets resulting in cell death [169]. Another mechanism of vorinostat induced cellular cytotoxicity is through the production of ROS and generation of DNA damage [168]. It has also been shown that inhibition of HDACs can impair the DNA-damage repair capacity of cancer cells [180, 181]. *Miller KM et al* demonstrated that HDAC1 and HDAC2 promote non-homologous end joining and their inhibition impaired DNA double strand break repair [181]. Lastly, some evidence suggests that vorinostat treatment can affect the intrinsic and extrinsic apoptotic pathways. Oral cancer cells were sensitized to TRAIL induced apoptosis through DR5 following treatment with vorinostat [182]. Vorinostat has been demonstrated to reduce the expression of mediators of the intrinsic apoptotic pathway, such as Bcl2 and Bcl-xL [183].

1.5 Activating Transcription Factors and AP1

The activating transcription factor (ATF) family consists of seven members (ATF1 to 7). These proteins all play a role in the cellular response to extracellular signals where they are involved in the transcriptional regulation of genes involved in stress response and survival; importantly in cellular responses to DNA damage [184]. ATF family members bind to the ATF/CRE consensus sequence (5'-TGACGTCA-3') and form homo- or hetero-dimers with other members of the ATF/CREB family of transcription factors to regulate target gene expression [184]. While these proteins belong to the same family, they do not share significant similarity in homology other than in their basic leucine zipper (bZIP) domains [184]. Diverse roles for each member of the ATF family have been demonstrated, but they have a common characteristic in their ability to respond to environmental signals and maintain cellular homeostasis [185]. For example, ATF2 and ATF3 are involved in the transcriptional control of stress response genes, while ATF6 is involved in upregulating genes involved in the endoplasmic reticulum stress response [185]. ATF1 is involved in mediating cellular response to intracellular cAMP concentrations, while ATF4 negatively regulates cAMP response elements [186, 187]. In order for ATF members to affect transcriptional regulation they form homo- or hetero-dimers with other members of the ATF/CREB family of transcription factors, including members from the ATF, JUN, FOS, and Maf families of proteins, and by doing so they form the AP-1 transcription factor [188].

A variety of roles for ATF proteins, as well as AP-1, have been demonstrated in cancer. The *ATF1* gene can be translocated with the *EWS* gene in Ewing-sarcoma, which results in the expression of the EWS/ATF1 fusion protein [189]. This fusion protein plays a role in tumor

formation and growth of Ewing sarcoma [189]. ATF4 expression has been demonstrated to be elevated in primary tumors and plays a role in tumor growth in xenograft models [190]. Overexpression or activation of ATF2 has been demonstrated to be involved in both oncogenic or a tumor suppressive roles [191]. Downregulation of ATF2 was demonstrated to inhibit the growth of pancreatic cancer cells and increase their sensitivity to gemcitabine, a nucleoside analogue [192]. ATF5 has been demonstrated to transcriptionally regulate the expression of mTOR, BCL2, and MCL1, genes involved in cell survival, and has therefore been proposed as a potential therapeutic target for the treatment of cancers [193]. Expression of ATF7 was positively correlated with overall survival and progression free survival in colorectal cancer patients [194]. ATF3 has also been demonstrated to play pro- and anti-tumorigenic roles in cancer, which is discussed in more detail in the following section. The function of these ATF proteins is dependent on context, as their interaction and dimerization with other proteins and the expression of potential binding partners which form the AP-1 transcription factor can dictate cellular function.

Activator protein 1 (AP-1) is a dimeric complex comprised of homo- or hetero-dimers of the JUN (c-jun, JunB, and JunD), FOS (c-Fos, FosB, Fra-1m and Fra-2), ATF (ATF2, ATF3, ATF4, JDP1, and JDP2), and MAF (c-Maf, MafA, MafB, MafG/F/K, and Nrl) protein families [188]. AP-1 is able to bind the promoters of genes at AP-1 binding sites (5′-TGA(C/G)TCA-3′) where it can induce transcription [195]. The AP-1 consensus binding sequences are also known as TPA and cAMP response elements. The various dimers that are formed alter AP-1's promoter-binding specificity. For example, JUN/JUN, JUN/FOS and JUN/FRA dimers bind with high affinity to the AP-1 binding sites, whereas JUN/ATF2 dimers predominantly bind to the cAMP

response elements (CRE) [195]. Generally, AP-1 dimers consisting of JUN proteins preferentially bind AP-1 sites (5′-TGA(C/G)TCA-3′) whereas dimers consisting ATF members bind CRE sites (5'-TGACGTCA-3') [196]. Activation of AP-1 relies on dimer composition, post-translational modifications, and protein interactions [195]. The dimer composition of AP-1 transcription factors is critical for determining its molecular function and therefore its specificity and regulation of its target genes [197]. AP-1 transcription factors have been implicated in a wide range of cellular processes and regulate genes involved in proliferation, apoptosis, differentiation, survival, migration, and transformation [198, 199]. AP-1 transcriptional activity and dimeric composition is strongly influenced by tissue-specific expression patterns of the individual proteins, as well as, their specific activation and expression mechanisms [196]. Dysregulation of the proteins that make up AP-1 have been linked to various diseases including cancer.

AP-1 expression is often deregulated in cancers as a result of dysregulated upstream signaling pathways regulating its activation and expression. Typically, AP-1 proteins are phosphorylated by upstream kinases, such as MAPK, which promote their activation and subsequent dimerization and DNA-binding. Varying roles for each protein that can make up the AP-1 dimer have been identified. For example, c-jun can promote cell proliferation through the upregulation of cyclinD1 and inhibition of p16 and p21, but c-jun can also mediate apoptosis in cells exposed to genotoxic stresses [200]. JunB inhibits cell proliferation through the upregulation of p16 and repression of cyclinD1 but can also be involved in the cell cycle progression through the upregulation of cyclinA [200, 201]. AP-1 has also been demonstrated to regulate the expression of a number of inflammatory cytokines, including interleukin (IL)-1, IL-2, interferon (IFN)-

gamma, and granulocyte-macrophage colony-stimulating factor (GMCSF) [188]. Lastly, an important target of the AP-1 transcription factor is the tumor suppressor p53, whose expression is modulated by a number of AP-1 proteins [200].

1.5.1 Activating Transcription Factor 3

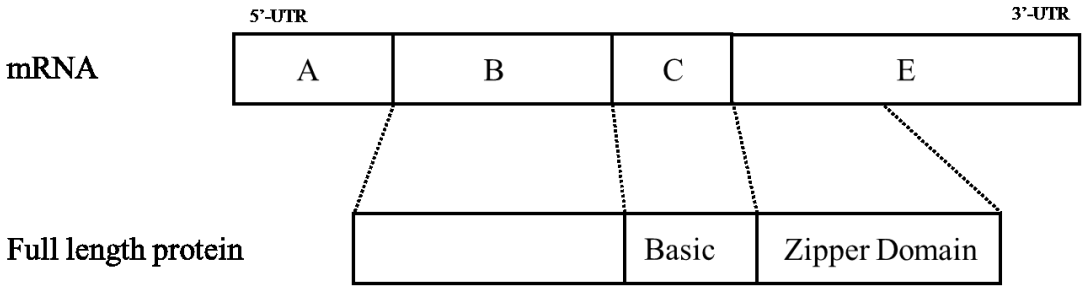
Activating transcription factor 3 (ATF3) is an adaptive response gene that is generally expressed at low levels in normal cells, but is strongly upregulated following a wide variety of cellular stresses, including hypoxia, inflammation, and genotoxic stress. *ATF3* is a member of the ATF/ cAMP responsive element binding (CREB) family of transcription factors. As discussed, the members of this transcription factor family are characterized by their ability to bind to the ATF/CRE consensus sequence, TGACGTCA, to regulate gene transcription. The *ATF3* gene is located at chromosome 1q32.3 and encodes a 181 amino acid protein with a molecular weight of ~22kDa protein [202]. The mRNA of *ATF3* contains four exons (denoted A, B, C, and E) which translate to the various structural domains of the ATF3 protein. Exon A encodes the 5'-untranslated region (UTR), exon B contains the start codon and N-terminal, exon C encodes most of the basic region, and exon E encodes the leucine zipper DNA binding domain and the 3'-UTR (Figure 3) [203]. An alternatively spliced isoform of ATF3 has also been described, ATF3ΔZip. This isoform contains an additional exon between exons C and E which results in a truncated protein due to the introduction of an in-frame stop codon. Therefore, this isoform lacks the leucine zipper and DNA-binding domain [203].

Studies investigating the regulation of the *ATF3* gene have identified a number of transcriptional regulatory sites in the 5'-flanking region, including an alternate promoter (P1) located 44kB upstream of the original promoter (P2) [204]. Transcription from either promoter results in the translation of identical proteins but occurs differentially depending on the stress stimuli. Transcriptional elements that regulate *ATF3* expression include a putative TATA box (P1 and P2) and transcription factor binding sites such as ATF/CRE, Activating Protien-1 (AP1), NFkB on P1 and P2, and p53 and E2F on P2 [204].

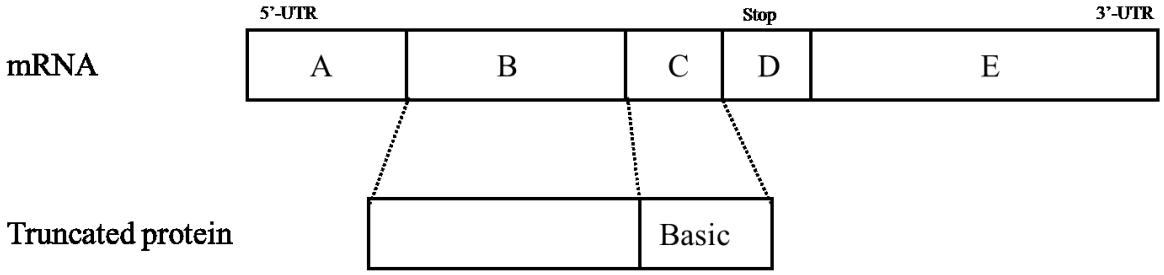
ATF3 acts as an activator or repressor of transcription through its ability to heterodimerize with other members of the ATF/CREB family of transcription factors or as a homodimer occurring through interactions with the leucine zipper domain of these proteins [205]. The function of ATF3 heterodimers has been demonstrated to be context dependent based on binding partner and promoter context, while the ATF3 homodimer acts as a transcriptional repressor [205]. Most notable is that ATF3 has been identified as part of the prevalent AP1 transcription factor and can heterodimerize with proteins that belong to the Jun, Fos, and ATF basic region-leucine zipper (bZIP) subfamilies where it mostly acts as a transcriptional activator [206].

Figure 3: Schematic of ATF3 mRNA and protein. Exons A, B, C, and E form a full length protein including a basic DNA binding region and a ZIP dimerization domain. ATF3ΔZip is encoded by exons A, B, C, D, and E where exon D encodes a stop codon resulting in a truncated protein. The ATF3ΔZip protein cannot dimerize with other proteins and cannot properly bind DNA. Adapted and modified from [203].

ATF3



ATF3ΔZip



1.5.2 Mechanisms of ATF3 Induction

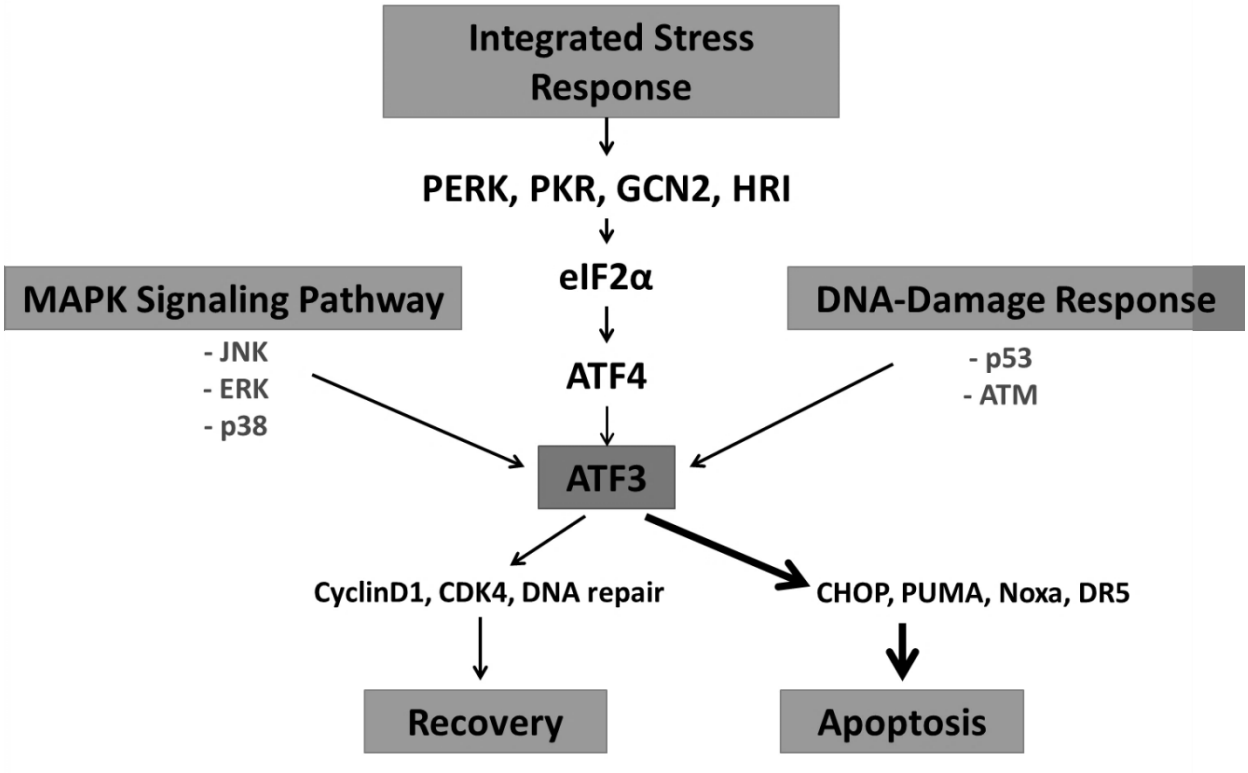
ATF3 expression is upregulated by a wide variety of cellular stresses, but not all of the molecular mechanisms involved in regulating its induction are well characterized. Upregulation of ATF3 has best been characterized as part of the Integrated Stress Response (ISR). The ISR is initiated through the activation of eIF2 (eukaryotic initiation factor 2) kinases [207]. Four eIF2 kinases have been identified; RNA-dependent protein kinase (PKR), PKR-like ER kinase (PERK), general control non-derepressible-2 (GCN2), and haem-regulated inhibitor (HRI) [207]. These kinases are activated upon varying stresses; GCN2 - amino acid deprivation; PKR- viral infection; PERK- unfolded proteins; and HRI- haem deprivation/oxidative stress. All four of these kinases phosphorylate eIF2a which results in the shutdown of protein translation except for transcripts that contain upstream open reading frames, such as ATF4 which can regulate the upregulation of ATF3 [207]. Drugs that induce the ISR can readily induce ATF3 including thapsigargin and lovastatin [208].

The MAPK signaling pathways have also been demonstrated to regulate ATF3 expression. Our laboratory demonstrated that MAPK signaling pathways are responsible for ATF3 induction following cisplatin treatment [209]. Inhibition of the three main branches of the MAPK signaling pathways (JNK, ERK1/2, and p38) resulted in the attenuation of cisplatin induced ATF3 expression in a variety of tumor derived cell lines [209]. Depending on the stress stimuli, a context dependent role for which MAPK pathway regulates ATF3 expression has been observed. *Lu D et al* demonstrated that p38, and not the other MAPK pathways, drives ATF3 induction following treatment with anisomycin [210]. Similarly, *Park EJ et al* demonstrated that

ERK regulates ATF3 expression following doxorubicin treatment in kidney proximal tubule cells [211]. Our group demonstrated that JNK was responsible for ATF3 induction following cisplatin treatment in the Calu6 and H23 NSCLC derived cell lines [212].

Other signaling pathways have been implicated in mediating ATF3 expression following cellular stress. *ATF3* is a target gene of p53, an important regulator of cellular response to DNA damage [213]. UV-irradiation or treatment with MG132, a proteasome inhibitor, enhanced ATF3 expression in a p53 dependent manner and through p53 binding to the ATF3 promoter [214]. In prostate cancer cells, the Kruppel-like factor (KLF6) binds to the ATF3 promoter and activates its transcription, and this induction of ATF3 was associated with increased apoptosis [215]. Additionally, the transcription factor early growth response (EGR1), which acts as a tumor suppressor, can regulate ATF3 expression in colorectal cancer cells [216]. ATF3 can also be induced following the activation of the DDR following the activation of Ataxia-Telangiectasia Mutated (ATM) [217]. Furthermore, a number of investigations of the ATF3 promoter has demonstrated binding and activity of the transcription factors EGR1, KLF6 and p53 [218].

Figure 4: Most common pathways involved in ATF3 induction following cellular stress. ATF3 expression has best been shown to be regulated through the integrated stress response, where activation of eIF2 kinases lead to the phosphorylation of eIF2α causing the upregulation of ATF4 and subsequently ATF3. All three branches of the MAPK signaling pathway, JNK, ERK, and p38, have been demonstrated to be involved in ATF3 upregulation. Similarly, some components of the DNA-damage response, such as p53 and ATM, have also been shown to regulate ATF3 expression. Once upregulated ATF3 has a dual role in mediating the cellular stress response, where it has been shown to upregulate genes involved in cell recovery. When upregulated expression levels of ATF3 are sustained it results in the upregulation of various apoptotic effector proteins such as CHOP, PUMA, and Noxa.


Integrated Stress Response
PERK, PKR, GCN2, HRI
eIF2α
MAPK Signaling Pathway
- JNK
- ERK
- p38
DNA-Damage Response
- p53
- ATM
ATF4
ATF3
CyclinD1, CDK4, DNA repair
CHOP, PUMA, Noxa, DR5
Recovery
Apoptosis

1.5.3 ATF3 as a Mediator of the Cellular Stress Response

Following ATF3 upregulation, it has been demonstrated to play a dual role in mediating cell stress responses. ATF3 upregulation can lead to cell cycle arrest which provides an opportunity for the cell to recover from the stress. If the cell is unable to overcome this stress, sustained and elevated expression of ATF3 can lead to cellular apoptosis. *Lu D et al* demonstrated that ATF3 binds the cyclin D1 promoter to repress its expression and subsequently promoted cell cycle arrest [219]. In chondrocytes, upregulation of ATF3 was also demonstrated to downregulate cyclin D1 and cyclin A expression [220]. In addition to being able to target cyclin D1, ATF3 has also been demonstrated to regulate inhibitor of differentiation (Id-1), which negatively regulates cell cycle exit [221]. In esophageal squamous cell carcinoma cell lines, over-expression of ATF3 resulted in repression of cyclin D1 and Id-1 and reduced cell proliferation [222].

ATF3 can also mediate cell death through the upregulation of CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP), also known as growth arrest and DNA-damage inducible gene 153 (GADD153) [223, 224]. CHOP is one of the apoptotic effector proteins of the ISR [223]. The pro-apoptotic role of CHOP results from its ability to affect expression of proteins involved in the intrinsic apoptotic response [225, 226]. CHOP down-regulates the expression of the anti-apoptotic protein, Bcl-2, sensitizing cells to cell death [226]. CHOP has also been linked to the upregulation of Bim, a BH-3 only protein involved in mediating cell death through the activation of Bax and Bak [225]. Knockdown of CHOP in SH-SY5Y cells resulted in decreased

Bim as well as another BH-3 only protein, PUMA expression [227]. In addition to CHOP, ATF3 has also been demonstrated to directly regulate the expression of the Bcl-2 family protein Noxa, which promotes apoptosis through the binding of the inhibition of the anti-apoptotic protein Mcl-1 [228].

ATF3 has also been demonstrated to mediate the cell stress response through its interaction with p53. *Yan C et al* demonstrated that ATF3 can stabilize p53 expression and activity by preventing its MDM2 mediated ubiquitination and subsequent degradation [213]. *Deng S et al* demonstrated that ATF3 mediated the upregulation of p53 expression following treatment with the topoisomerase inhibitor dexrazoxane [217]. Loss of ATF3 negatively affects the transcriptional activity of p53 and impaired its ability to induce apoptosis in ATF3-/- MEFs exposed to ionizing radiation [229].

1.5.4 Role in Immunity

ATF3 can also play an important role in immune regulation. ATF3 was identified as a key transcript upregulated by the Toll-like receptor 3 (TLR3), TLR2, TLR4, TLR7 and TLR9 [230]. TLRs are key components for the recognition of pathogens and initiation of the signaling cascades for the expression of various inflammatory cytokines, chemokines and co-stimulatory molecules that are responsible for the activation of adaptive immune responses [202]. The loss of ATF3 in murine bone marrow macrophages (BMM) resulted in elevated production of interleukin-6 (IL6) and IL-12p40 cytokines [230], including following LPS treatment, a TLR4 agonist [231]. ATF3 was demonstrated to bind to the promoters of IL-6 and IL-12b to

functionally repress their expression [231]. *In vivo* studies in ATF3 knockout mice showed elevated levels of IL-6, IL-12b and TNFα. These mice also demonstrated a higher mortality due to endotoxic shock compared to wild-type mice [231]. These studies demonstrated that ATF3 plays a role in repressing the cytokine/chemokine production and as a regulator of immunity against foreign pathogens [202, 231]. In addition to macrophages, ATF3 can also repress the production of cytokines/chemokines in other immune cells, including natural killer (NK) cells, CD4+T cells, and mast cells [232]. An example of the context dependent regulation of cytokine production by ATF3 is that it represses TNFα expression in macrophages [230] but not in NK cells [233]. Furthermore, ATF3 has also been demonstrated to regulate cytokine/chemokine production in non-immune cells, however in this context ATF3 upregulates their expression. *Yin X et al* demonstrated that ATF3 upregulates the expression of IL-1β, IL-6, TNFα, and monocyte chemotactic protein 1 (MCP1) in pancreatic islet cells upon various stress stimulations [234]. Since IL-1β, IL-6, TNFα can induce the expression of ATF3, this suggest that ATF3 is involved in a positive-feedback loop to amplify cytokine/chemokine production, which is contrary to its role in immune cells [232]. Together these studies identify an important and context dependent role for ATF3 in immune response modulation.

1.5.5 Role in Cancer

The role of ATF3 in cancer is not well characterized with studies demonstrating pro- or anti-tumorigenic functions dependent on cell context. This is best exemplified in a study by *Yin X et al* which demonstrated a dichotomous role for ATF3 in breast cancer. They demonstrated that ATF3 overexpression protected the malignant breast cancer cell line, MCF10A1a, from

apoptosis and potentiated their metastatic potential [235]. Conversely, in the non-malignant MCF10a cells, ATF3 enhanced cellular apoptosis.

Several studies have provided evidence for the role of ATF3 as an oncogene. ATF3 is overexpressed in multiple human tumor types. In breast cancer, the chromosomal locus of ATF3 is amplified and correlates with elevated ATF3 expression in these tumors [235]. According to publically available data (cBioPortal), ATF3 was found to be amplified in 354 of 5944 (5.9%) breast cancer patients, and 119 of 6984 (1.7%) NSCLC patients. Additionally, in malignant prostate cancer there is a correlation between higher ATF3 expression and indicators of poor prognosis [236]. Elevated ATF3 expression was also observed in lung tumor tissue and correlated with advanced tumor grade [237]. Further studies, examining the cellular function of ATF3 have also provided evidence for its role as an oncogene. In the prostate cancer cell line DU-145 overexpression of ATF3 resulted in increased proliferation [236] and overexpression of ATF3 in prostate cancer cell lines enhanced invasiveness and promoted lung metastasis *in vivo* [238]. In line with this data, knockdown of ATF3 in the colon cancer cell line HT29 inhibited cell invasion *in vitro* and inhibited tumor growth *in vivo* [239]. Furthermore, knockdown of ATF3 inhibited tumor growth of HT29 and CaCO2 xenografts and reduced liver metastasis [240]. Lastly, in a lung cancer cell lines ATF3 knockdown inhibited cell proliferation, cell cycle progression, and migration [237]. Together these studies suggest that ATF3 can play a pro-tumorigenic role in a number of cancers.

There have also been a number of studies that demonstrate a role for ATF3 as a potential tumor suppressor. ATF3 knockout mice develop spontaneous tumors more frequently than their wild-

type counterpart and died significantly earlier [229]. Loss of ATF3 expression results in increased cell proliferation and survival and promoted the progression of invasive tumors in a *PTEN* knockout mouse model of prostate cancer [241]. In a different prostate cancer mouse model, loss of ATF3 promotes hormone induced prostate carcinogenesis [242]. Also, ATF3 expression is down regulated in esophageal squamous cell carcinomas compared to healthy tissue and that this correlated with reduced overall survival [243].

Of importance to this study, there is increasing evidence to suggest that ATF3 plays an important role in mediating the cytotoxic effect of various anti-cancer agents. Our laboratory has demonstrated that ATF3 plays an important role in mediating the effect of cisplatin induced cytotoxicity in NSCLC [212]. Additionally we demonstrated that ATF3 is dysregulated in cisplatin-resistant NSCLC cell lines [212]. ATF3 expression can also be upregulated by other DNA damaging agents including topoisomerase inhibitors [217, 218]. In prostate cancer cells, ATF3 was demonstrated to mediate the cytotoxicity of the topoisomerase inhibitor, retigeric acid B [218]. Doxorubicin cytotoxicity has also been shown to be mediated by ATF3 in human kidney proximal tubule cells [211]. Camptothecin, another topoisomerase inhibitor, was demonstrated to upregulate ATF3 expression in colorectal cancer cells, and this was required for efficient upregulation of death receptor 5 (DR5) mediated apoptosis [244]. Other DNA-damaging agents, such as UV-irradiation, have also been demonstrated to have mechanisms of cytotoxicity dependent on ATF3. In addition to ATF3's role in mediating apoptosis following treatment with DNA-damaging agents, it has also been demonstrated to regulate the cytotoxicity of drugs such as digitoxin [245], indole-3-carbinol [246], and tolfenamic acid [180]. Taken

together, these findings suggest that ATF3 can regulate the cytotoxic effect of a variety of agents including DNA-damaging chemotherapies.

1.6 Novel Combination Based Therapies to Enhance Chemotherapy Response

Combination therapies have become part of the standard of care for the treatment of many cancers, as they provide the ability to target multiple cellular pathways in order to induce tumor cell death. Many combinations, such as the use of platins and taxol in NSCLC, have demonstrated improved efficacy in patients and increase patient survival. While these combination chemotherapy regimens have proved effective, many have been determined empirically and the molecular mechanisms underlying their functions are not well characterized. The mechanisms driving drug synergy are generally grouped into three categories. Parallel pathway inhibitors target different parallel cellular pathways that are essential for an observed phenotype including cellular proliferation or survival. A single signaling pathway can also be *"vertically" targeted*, with drugs inhibiting multiple steps in a signaling cascade to enhance its inhibition, for example the use of BRAF and MEK inhibitors in advanced-stage melanoma harboring $BRAF^{V600}$ mutations. In addition, the *bioavailability model* suggests that two drugs will be synergistic if one drug's action helps another drug's availability in the target cells [247]. A relatively novel synergistic model has recently been suggested that proteins that are involved in both cell recovery and apoptosis can be driven to apoptosis by targeting its expression to drive its apoptosis response highlighted by p53 y [248]. *Chen et al* demonstrated that the apoptotic response mediated by p53 is affected by alterations in p53 dynamics and elevated and sustained p53 expression drives its pro-apoptotic response in a dose-dependent manner [248]. This can be achieved by combining agents that induce p53 or through agents that affect its degradation.

Importantly, p53 is a significant inducer of ATF3 and both are regulated by MDM2 mediated degradation, therefore enhancing and sustaining ATF3 expression like p53 may drive cellular and in particular tumour cell apoptosis.

1.7 Rationale

DNA-damaging chemotherapeutics cisplatin and doxorubicin are representive of the two classes of the most important agents in cancer treatment extensively employed for the treatment of NSCLC and breast cancer, respectively. Unfortunately, their use can be limited due to significant toxicities and eventual acquired tumor resistance. Identifying key regulators of cisplatin and doxorubicin's cytotoxic effect will uncover novel rational therapeutic strategies enhancing their therapeutic efficacy. ATF3 is a stress inducible gene that we have demonstrated is involved in mediating the cytotoxicity of cisplatin [209]. Taken together with a variety of other laboratories' studies, ATF3 may represent a key mediator of other DNA-damaging treatment modalities. Uncovering the role of ATF3 in their cytotoxicity responses, may uncover ATF3 as a potential therapeutic target for the rational development of novel combination based therapies.

1.8 Hypothesis

ATF3 is a key regulator of cisplatin and doxorubicin induced cytotoxicity and represents a novel mediator of their cytotoxic effect. Additionally, the use of alternative ATF3 inducers in

combination with these drugs will enhance tumor cell cytotoxicity as sustained and elevated expression of ATF3 triggers cellular apoptosis.

1.9 Overall Objectives

To identify ATF3 as a therapeutic target and identify inducing agents that can enhance the cytotoxicity of clinically relevant DNA-damaging chemotherapies.

1.10 Specific Aims

1) Evaluate the role of ATF3 in regulating cisplatin and doxorubicin induced tumour cell cytotoxicity
2) Identification of novel inducers of ATF3 for potential combination therapeutic approaches
3) Evaluate the therapeutic potential of combining inducers of ATF3 with cisplatin and doxorubicin

Chapter 2: Material and Methods

2.1 Tissue Culture

The human NSCLC tumor-derived cell lines, Calu6, NCI-H23 (H23) and A549, the breast cancer cell lines, MCF7 and MDAMB231, and the prostate cancer cell line LNCaP and PC3 were obtained from the ATCC (Rockville, MD, USA). The ATF3 (-/-) and wildtype murine embryonic fibroblasts (MEFs) were generously provided to us by Dr. T. Hai (Ohio State University, Columbus, OH). Calu6 and H23 are two commonly employed NSCLC cell lines and models of this disease that both contain mutations for *TP53* and *KRAS*. These are common mutations found within NSCLC tumors that drive tumour growth [14]. The MCF7 and MDAMB231 are two commonly employed breast cancer lines that represent luminal and triple-negative breast cancer subtypes, respectively. The MCF7 cells are *TP53* wild-type while the MDAMB231 cells are mutated for this gene [249]. The Calu6, H23, A549, MCF7, PC3 and MEF cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and the MDAMB231 were maintained in low-glucose DMEM (Mediatech, Manasas, VA). LNCaP cells were maintained in Roswell Park Memorial Institute medium (RPMI). All media was supplemented with 10% fetal bovine serum (Medicorp, Montreal QC) and 100U/ml penicillin/streptomycin (ThermoFisher Scientific, Waltham, MA). Incubation of the cells occurred at 37^{o}C and 5% CO_2. Passaging of cells involved washing with PBS and detachment using Trypsin EDTA 1X (Cellgro, Manassas, VA). Cell counts were performed using the Vi-Cell XR cell viability analyzer (Beckman Coulter, California, USA).

ATF3 knockout in the MCF7 cell line was performed by lentiviral transduction of CRISPR/Cas9 using an optimized ATF3 CRISPR guide RNA purchased from GenScript (Piscataway, NJ). Lentivirus expressing the CRISPR/Cas9 and guide-RNA were generated through transfection of 293T packaging cells cultured in DMEM supplemented with 10% FBS with the two packaging plasmids, one encoding Rev and one encoding Gag and Pol and the transfer plasmid encoding the CRISPR/cas9 and gRNA (Addgene, 3rd Generation Lentiviral Packaging). Supernatant containing packaged virus was collected 48 hours following transfection and purified through centrifugation and filtration. Purified virus was used to infect the MCF7 cells through the addition of 500ul of the purified collected supernatant containing the lentivirus with 4ug/ml polybrene in a final volume of 2mL of DMEM supplemented with 10%FBS and 100U/ml penicillin/streptomycin. Following 24 hours of infection, MCF7 cells were selected with puromycin for 5 days at 1.5ug/ml and the surviving fraction was expanded and single cell colonies were collected and screened for ATF3 knockout by Western blot.

To derive the cisplatin resistant Calu6 and H23 sublines, cells were plated to 80 % confluence in a 25 cm^2 culture dish and treated with 2mg/mL cisplatin for 21 days replacing the media and drug every 2-3 days[250]. Following the 21 day treatment, the surviving fraction (approximately 10^{-6} cells) was collected and sub clones derived from single cells were selected and expanded. Sub clones (denoted as cisR) were subsequently propagated for 20 passages and their sensitivity to cisplatin was measured through MTT assay.

For Calu6cisR1 transfections, cells were plated at a density of 3 x 10^5 in a 6-well plate and transfected with 2ug of the JNK1a1-expressing plasmid (Addgene, Plasmid #13,798) [251] using

FuGENE HD Transfection Reagent (Roche, Mississauga, ON) as per manufacturer's protocol. After 24 hours the culture medium was removed and replaced with media containing cisplatin for an additional 24 hours. Western blot was performed to measure expression of activated JNK through its downstream target p-cjun. For the MTT assays, cells were plated at 5000 cells/well in a 96 well plate and transfected with 2ug of the JNK1a1 plasmid. 24 hours following transfection the media was changed and the cells were treated with cisplatin for 48 hours. MTT assay was performed to determine cell viability.

Cisplatin, carboplatin, doxorubicin, and docetaxel were obtained from the pharmacy at the Ottawa Hospital Cancer Centre. Chemical inhibitors for JNK (SP600125), ERK1/2 (U0126), p38 (SB203580), and ATM (KU55933) were purchased from Selleck Chem (Houston, TX) and stocks were prepared in DMSO. Vorinostat was purchased from Calbiochem (Gibbstown, NJ) and trifluridine and 6-mercaptopurine were purchased from Sigma-Aldrich (St. Louis, MO).

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

Human breast and NSCLC cancer cells were seeded at a density of 5000 cells/ 50µL in flat bottom 96-well plates (Costar, Corning, NY) and incubated overnight to allow cells to adhere. MEFs were seeded at a density of 2500 cells/ 50µL. The following day, drug treatments were administered up to a final volume of 100µL. For MTT analysis, 42µL of a 5mg/mL solution of MTT tetrazolium substrate (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline was added and incubated for up to 2 hours at 37^{o}C. The resulting violet formazan precipitate was solubilized by the addition of 84µL of 0.01M HCl in 10%SDS (Sigma-Aldrich, St. Louis, MO)

at 37^{o}C overnight. Plates were analyzed on a microplate reader at 570nm (Synergy Mx Monochromator-Based Multi-Mode Microplate Reader, Biotek Instruments, Winooski, VT)

2.3 Western Blotting

Cells were plated at 0.8 × 10^{6}/60-mm dish and incubated overnight followed by drug treatment with the indicated drug for 24, 48, or 72 hours. Protein samples were extracted in RIPA buffer (50 mM Tris-CL pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1% Triton-X-100, 0.25% sodium deoxycholate, 0.1% SDS) containing the protease inhibitors 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerolphosphate, and 1× Protease Inhibitor Cocktail (Sigma-Aldrich). Protein concentrations were determined using the BCA protein quantification assay (ThermoFisher Scientific) following manufacturer's instructions. Western blots were performed as previously described [209]. Briefly, protein samples were run on 12% acrylamide gels in electrophoresis running buffer (25mM Tris-HCL, 192mM glycine, 0.1%SDS) at constant voltage (120V) for until loading dye front reached the bottom of the gel (approximately 1.5 hours). Proteins were then transferred to PVDF membrane at a constant voltage of 100V for 1 hour in transfer buffer (25mM Tris-HCL, 192mM glycine, 20% methanol). Membranes were blocked for 1 hour in 5% BSA in Tris-Buffered Saline with 0.1% Tween-20 (v/v) (TBST). Membranes were incubated with primary antibodies overnight at 4^{o}C on a rocker. The following day membranes were washed (three 5 min washes) and were incubated with the appropriate HRP-tagged secondary antibody for 1 hour at room temperature (anti-rabbit (1:3000) and anti-mouse (1:3000), Jackson ImmunoResearch, West Grove, PA, USA). Following incubation with the secondary antibody, membranes were washed (three 5 minute washes) and visualized using

the Clarity Western ECL substrate (BioRad, Mississauga ON) and developed using the Syngene Bio-Imaging System (Syngene, Frederick, MD). All antibodies were prepared in 5% BSA in TBST. Antibodies specific for ATF3 were purchased from Santa Cruz Biotechnology and were used at a dilution of 1:500 and were detected using anti-mouse secondary (Santa Cruz, CA); Actin from Sigma (1:10000 dilution; anti-mouse secondary); ERK (1:1000 dilution; anti-rabbit secondary), phospho-ERK (Tyr204) (1:1000; anti-mouse secondary), cJun (1:1000; anti-rabbit secondary), phospho-Jun (Ser73) (1:1000; anti-rabbit secondary), Hsp27 (1:1000; anti-mouse secondary), phospho-hsp27 (Ser78) (1:1000; anti-rabbit secondary), PARP (1:1000; anti-rabbit secondary) from Cell Signaling Technology (Beverly, MA); and γH2A.X (Ser139) (1:1000; anti-mouse secondary) from Millipore.

2.4 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Cells plated at 0.6×10^6 cells per 6-cm dish were incubated at 37°C overnight and then treated with doxorubicin for 24 hours. Total RNA was extracted from cell samples using Trizol (Thermo Scientific). The concentration of RNA was quantified using Take3 Micro-Volume plate and BioTek Synergy MX plate reader and analyzed using Gen5 software (BioTek, Winooski, VT). One microgram of total RNA was reverse-transcribed to complementary DNA for RT-qPCR using a High Capacity cDNA reverse transcription kit (Applied Biosystems) in an ABI Thermal Cycler (Applied Biosystems, Foster City, USA). Real-time PCR was performed using Taq Man Gene Expression Assay Primer/Probes for ATF3 (HS00231069), DDIT3 (HS00358796) and the housekeeping gene human GAPDH (HS4333764-F) as per manufacturer's instructions (Applied Biosystems). The total PCR reaction volume of 20µl contained 2µl of cDNA, 1µl TaqMan Gene

Expression Assay Primer/Probe (20x), 10µl of TaqMan Universal PCR Master Mix (2x) (Applied Biosystems, 4304437) and 7µl of RNase-free water. The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification. Three independent experiments were performed to determine the average gene expression and standard deviation.

2.5 *Ex-Vivo* Tissue Samples

Tumor samples were obtained from patients undergoing surgical resection for their lung, breast and ovarian tumors at the Ottawa Hospital (Ottawa Hospital Research Ethics Board; Protocol # 20120559-01H). For lung specimens, adjacent normal tissue was also collected from patients who underwent lobectomy. Areas containing tumor were identified by routine pathological gross examination. Tissue was collected in DMEM supplemented with 10% FBS, 100U/ml penicillin/streptomycin, and 0.25ug/ml Amphotericin B. Tumor tissue was processed using a sterile 2mm biopsy punch followed by slicing with a scalpel to obtain cores that were approximately 2x2x1 mm in size. Cores were randomized and distributed, 3 per well in a 24-well plate containing supplemented with 10% FBS, 100U/ml penicillin/streptomycin, and 0.25ug/ml Amphotericin B (Sigma- Aldrich). Cores were drug-treated for 48 hours with doxorubicin and then collected for RNA processing and RT-qPCR for ATF3 mRNA expression. Treatments were done in triplicate (3 wells and 3 cores/well) per condition. For RNA collection, cores were homogenized in Trizol using a hand-held homogenizer (Fisher Scientific). RNA extraction was carried out following the manufacturer's protocol for Trizol and RT-qPCR was performed for ATF3 expression using Taq Man Gene Expression Assay Primer/Probes for ATF3

and GAPDH as an endogenous control. Expression of ATF3 per each well per condition was measured to determine the average gene expression and standard deviation.

2.6 RNA Sequencing Transcriptome Analysis

RNA was collected from Calu6, H23, and their respective cisR1 sublines using the RNeasy Isolation Kit following the manufacturer's guidelines (Qiagen, Frederick, MD). Messenger RNA expression profiling was performed using NuGen reagents (www.nugeninc.com). Following amplification, libraries compatible with Illumina Next Generation Sequencing methods were prepared using the Ovation Ultra Low Library Prep Kit (NuGen, San Carlos, CA). The quality of the library was determined using a Bioanalyzer 2100 (Agilent Technologies, Mississauga, ON). Kappa Library Quant Kits (KappaBiosystems, Wilmington, MA) were used for library quantitation. Cluster generation and 2 x 36 bp paired-end sequencing was performed over a single lane using the Illumina GAIIx or HiSeq Genome Analyzer workstation. We focused on differentially expressed genes that were cisplatin dependent, limiting our analysis to only cisplatin-treated cells. The RNA-seq analysis was performed by StemCore (Ottawa Hospital Research Institute) using an Illumina GAIIx platform generating 40 million sequencing reads per sample with 89% to 90% of reads mapping back to the genome. TopHat/Cufflink pipeline was used for mapping reads to the genome and quantification of gene expression [252]. Computational assessments were conducted by the Ottawa Bioinformatics Core Facility. Following analysis Gene Ontology enrichment analysis was performed using PANTHER (www.pantherdb.org) [253].

2.7 High Throughput Library Screen

A chemical library of 1200 FDA-approved compounds (Prestwick Chemical, Illkirch, France) was used to treat wild-type and ATF3-/- MEFs. All compounds were provided at an initial stock concentration of 10mM in DMSO and were used at a final concentration of 5µM. Wild-type and $ATF3^{-/-}$ MEFs were plated at 2500 cell/well in 96 well plates. They were treated for 48 hours with 5uM of the library compound (single well) using the Biotek Precision Pipetting system. Cytotoxicity to the test compounds was assessed by MTT assay following 48 hour treatment. A difference in response between the wild-type and ATF3-/- MEFs of greater than 20% was considered a "hit".

Combination screens employing the 1200-FDA approved library were performed to identify enhancers of cisplatin and doxorubicin cytotoxicity. Calu6 and MCF7 cells were plated at 5000 cells/well in 96 well plates. Calu6 cells were treated for 24 hours with the library compounds at 1uM followed by 48 hour treatment with 0.4ug/mL cisplatin. MCF7 cells were treated with the library compounds at 1uM for 24 hours followed by 48 hour treatment with 10nM doxorubicin. The concentration of cisplatin and doxorubicin used was the dose that resulted in approximately 20% cell death following treatment. Following treatment MTT assays were performed to assess cell viability. A "hit" was defined as a compound that could enhance cisplatin or doxorubicin cytotoxicity by greater than 20% compared to the library compound and chemotherapy alone.

2.8 IC50 and Combination Index

The combination index of the drug combination studies was calculated using the Chou-Talay Method [254]. The data was inputted to the Calcusyn Software (Biosoft, Cambridge, UK) and combination indexes (CI) vs. fraction affect plots were generated. A CI value ≤ 1 is synergistic, a value equal to 1 is additive, and a value ≥ 1 is non-synergistic. IC50 values were determined through Calcusyn.

2.9 Statistical Analysis

The presented graphical data and statistical analysis was plotted and analyzed using Graphpad Prism (Version 6.0). Significance between columns for RT-qPCR data was determined by one-way ANOVA using Bonferroni multiple comparison test. For MTT viability curves, significance was determined by two-way ANOVA employing a Bonferroni multiple comparison test. For all test, the criteria for significance was $p < 0.05$. The combination effect of doxorubicin with vorinostat was calculated using CalcuSyn (Biosoft, Cambridge, UK). Combination index (CI) values were graphed on fraction affected-CI (Fa-CI) plots. A $CI < 1$ is a synergistic interaction, $CI = 1$ is additive, and $CI > 1$ is antagonistic.

Chapter 3: Results

In the following chapter experiments for Figures 5 and 6 were performed by Jair Bar and Jennifer Hanson. RT-qPCR for Figure 7 was completed by Mohamed Shaad Hasim and Tabassom Baghai. All experiments of Figure 8 were completed by Mohamed Shaad Hasim. MTT assays for cisplatin, carboplatin, and docetaxel of Figure 9 were performed by Tabassom Baghai. MTT for doxorubicin of Figure 9 was done by Mohamed Shaad Hasim. Western blots of Figure 9 were completed by Jennifer Hanson. All experiments of Figure 10 were performed by Tabassom Baghai. The library screens done in the MEFs presented in Table 1 was performed by Mohamed Shaad Hasim. The library screen presented in Table 2 was performed by Stephanie Reid.

3.1 Induction of Activating Transcription Factor 3 Is Associated with Cisplatin Responsiveness in Non-Small Cell Lung Carcinoma Cells

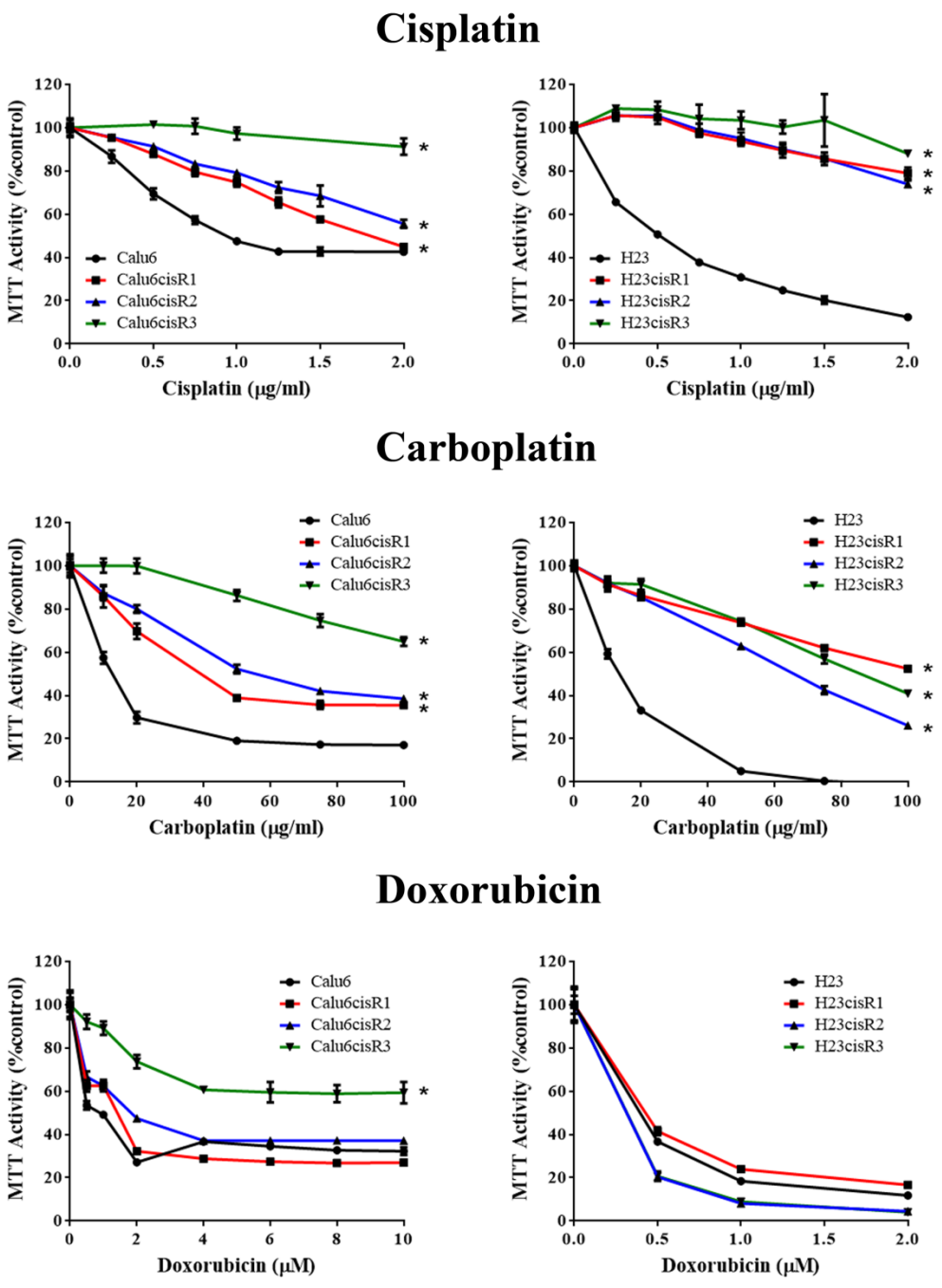
3.1.1 Non-Small Cell Lung Cancer Model of Cisplatin Resistance

In this study, we predominately employed the *in vitro* human lung carcinoma Calu6 and NCI-H23 (H23) cell line models. Both cell lines were derived from NSCLC adenocarcinomas and harbour mutations in *KRAS* and *p53*, which are common to this NSCLC tumor type [255, 256]. Initially, we developed cisplatin resistant sub-clones from both of these cell lines (denoted as Calu6cisR1-3 and H23cisR1-3) through prolonged treatment of 2µg/mL cisplatin until a fraction of ~10^{-6} cells remained [250]. Surviving colonies were isolated and cultured for 20 passages and

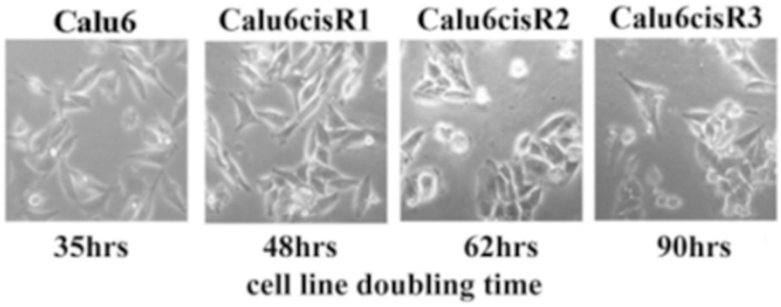
their sensitivities to cisplatin, carboplatin, and doxorubicin were assessed by the MTT cell viability assay (Figure 5A). The parental Calu6 and H23 cell lines demonstrated significant sensitivity to treatment with all three DNA-damaging chemotherapeutics that included cisplatin, carboplatin and doxorubicin. The Calu6cisR sub-clones demonstrated varying levels of resistance to treatment with cisplatin and carboplatin while the H23cisR sub-clones showed a more consistent resistance response. All resistant clones, with the exception of the Calu6cisR3, had similar levels of cytotoxicity to their parental cell line when treated with doxorubicin, demonstrating that the resistant phenotypes showed specificity to platins and did not affect responsiveness to doxorubicin. To further asses the characteristics of the resistant cell lines; we measured the proliferation rate through trypan blue exclusion assay over the course of 200 hours to determine doubling time (Figure 5B). The H23cisR clones demonstrated similar growth kinetics compared to their parental counterparts, while the Calu6cisR clones displayed a significantly slower growth rate particularly the Calu6cisR3 which correlated with its resistance to the tested DNA-damaging chemotherapies. Subsequent experiments focused on the Calu6cisR1 and H23cisR1 as representative sub-clones in comparison to their parental cell lines.

Figure 5: (A) MTT analysis of Calu6, Calu6cisR1-3, H23, and H23cisR1-3 following 48-hour treatments with cisplatin, carboplatin, and doxorubicin. Cisplatin and carboplatin treatments showed reduced cytotoxicity in the Calu6cisR1-3 and H23cisR1 compared to their parental cell lines. With the exception of Calu6cisR3, response to doxorubicin treatment was similar in the parental and cisR1 sublines. *Significant difference in the viability curves of the cisR sublines compared to their parental counterparts (Pb .05). (B) No significant morphological differences were observed between the parental and resistant sublines (phase microscopy); however, proliferation rates as determined by cell doubling times were reduced in the Calu6cisR sublines, and this was most pronounced in the Calu6cisR3 cell line. (C) IC50 concentrations for cisplatin resistant clones for cisplatin, carboplatin, and doxorubicin.

A


Cisplatin
MTT Activity (%control)
Cisplatin (μg/ml)
Calu6
Calu6cisR1
Calu6cisR2
Calu6cisR3
H23
H23cisR1
H23cisR2
H23cisR3
Carboplatin
Carboplatin (μg/ml)
Doxorubicin
Doxorubicin (μM)

B


Calu6
Calu6cisR1
Calu6cisR2
Calu6cisR3
35hrs
48hrs
62hrs
90hrs
cell line doubling time

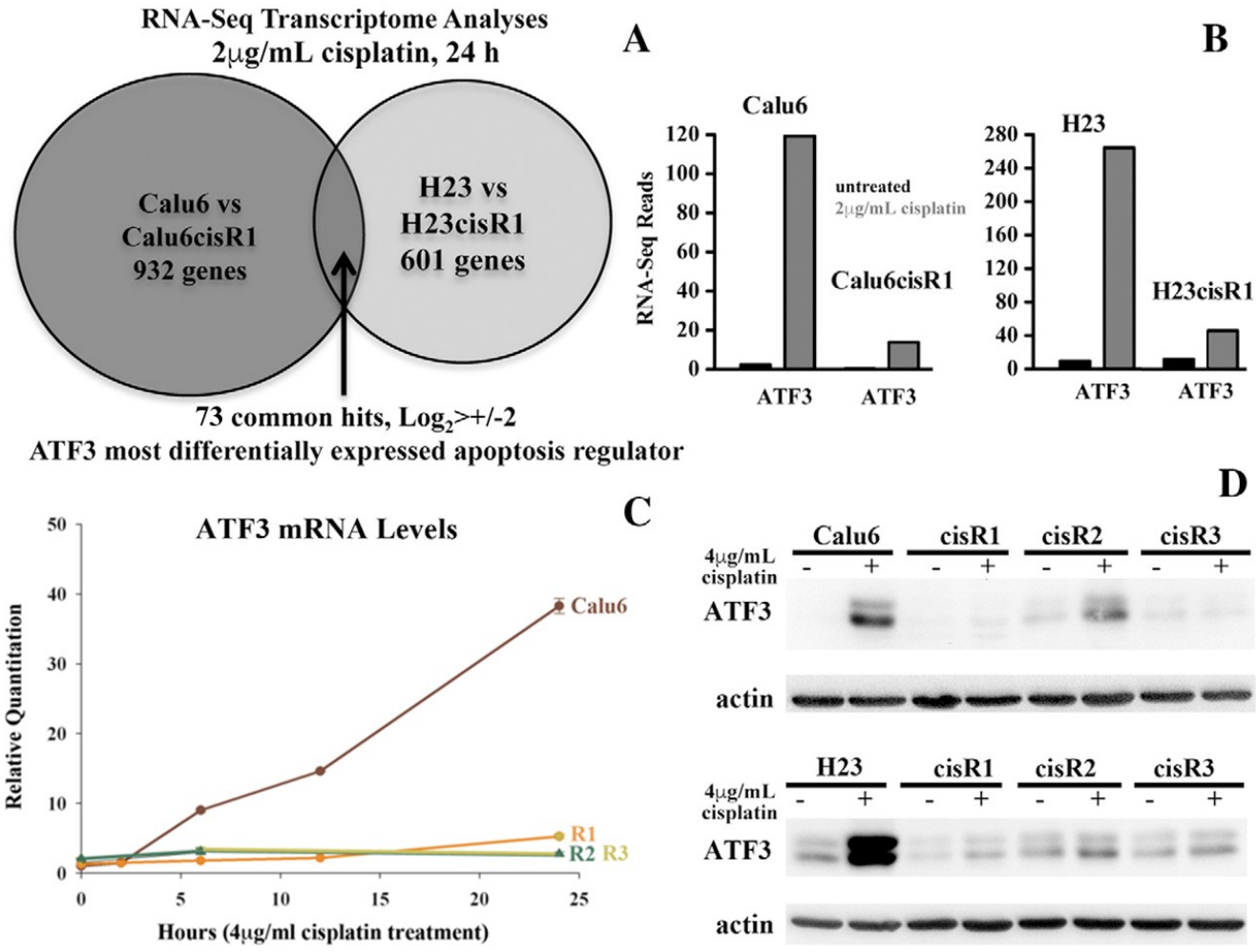
C

Cisplatin Resistant Cell Lines				
	IC50, MTT analysis, 48hr			
		Cisplatin (μg/ml)	Carboplatin (μg/ml)	Doxorubicin (μM)
Calu6	Parental	1.01	10.93	0.51
	Calu6 CisR1	2.20	45.15	1.19
	Calu6 CisR2	2.98	60.19	1.92
	Calu6 CisR3	8.57	132.73	11.11
H23	Parental	0.47	13.31	0.39
	H23 CisR1	5.18	95.55	0.44
	H23 CisR2	3.30	59.48	0.31
	H23 CisR3	4.76	80.34	0.32

3.1.2 ATF3 Expression is Dysregulated in Cisplatin-Resistant NSCLC Cell Lines

Currently, the focus of most studies is to identify genomic or transcriptome alterations at baseline to predict tumour responsiveness to treatments. A number of DNA damage response genes have been shown to be predictive of responsiveness to platins, however, they lack the sensitivity and robustness to be employed in the clinic [257]. New approaches are needed to identify both key regulators of platin responsiveness and their potential as predictive biomarkers. The potential of differential on treatment changes in expression between sensitive and resistant NSCLC tumour cells may uncover such regulators of cisplatin responsiveness. In order to identify such differentially expressed genes we performed RNA-seq full transcriptome analysis comparing the cisplatin-sensitive parental Calu6 and H23 cell lines with their paired resistant cisR1 clones. We compared the expression levels of the parental cell line to the cisR1 clone treated with 2μg/mL cisplatin. This allowed for us to identify cisplatin dependent differentially expressed genes. We identified 932 and 601 genes that were differentially expressed following cisplatin treatments of at least 4 fold between the Calu6 versus Calu6cisR1 and H23 versus H23cisR1, respectively. A comparison of the differentially regulated genes identified 73 genes in common between the two cell lines (Figure 6A). A gene list of the 73 common differentially expressed genes is presented in appendix table 1 along with GO term analysis (appendix table 2). *ATF3* was found to be the most significantly upregulated apoptosis regulatory gene in both parental cell lines that was not significantly induced in the resistant sub-clones. Validation through RT-qPCR confirmed that cisplatin treatment failed to induce ATF3 expression in the resistant sub-clones compared to their parental counterparts (Figure 6C). Western blot analysis also confirmed that cisplatin treatment significantly induced ATF3 expression in both Calu6 and H23 parental cell lines but not in the resistant cisR sub-clones (Figure 6D).

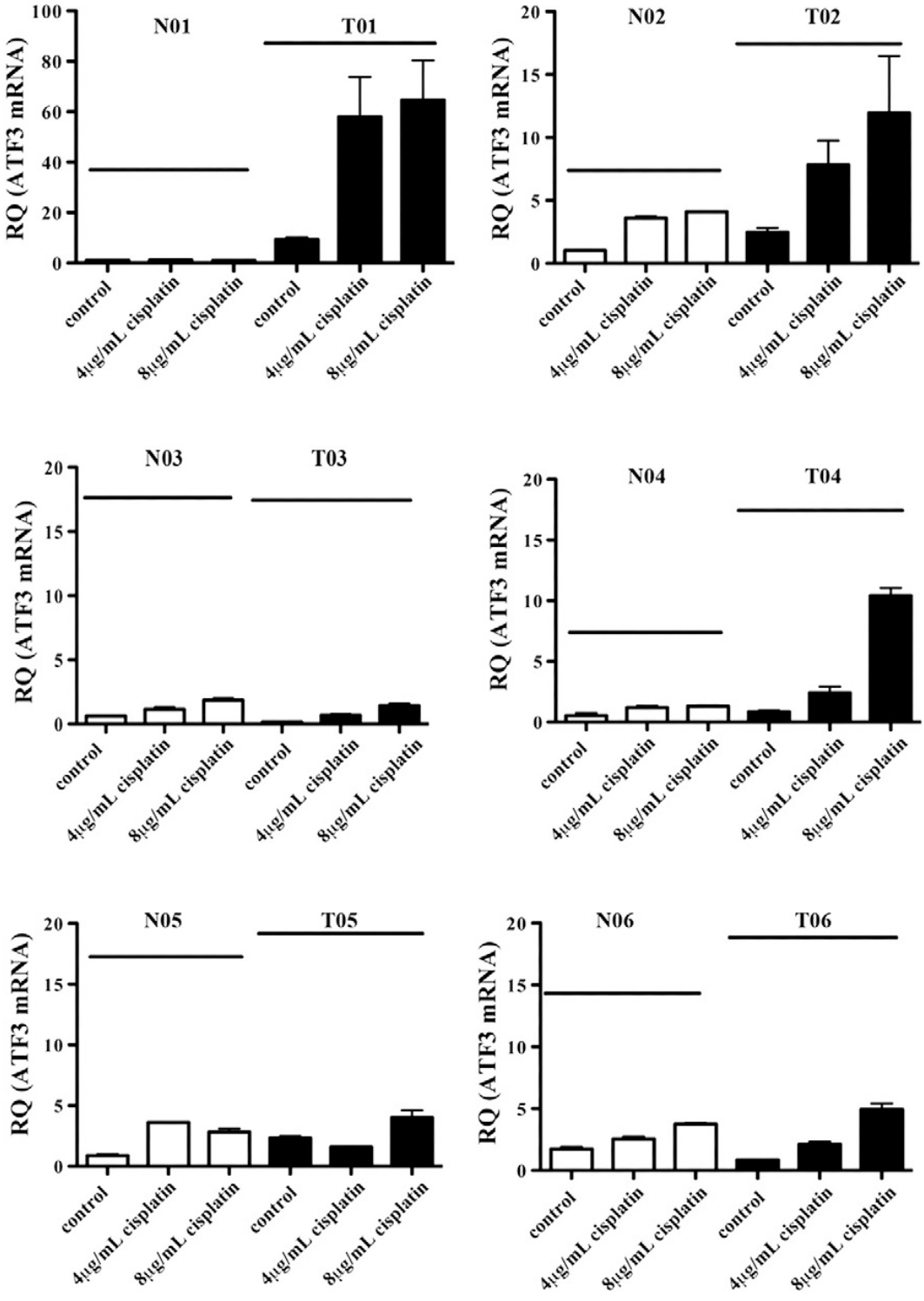
Figure 6: (A) RNA-seq analysis of Calu6 and H23 parental versus their respective cisR1 resistant sub-clones identified 42 commonly differentially expressed genes in both cell lines following 2-μg/ml cisplatin treatment for 24 hours. (B) The cellular stress response gene ATF3 was differentially induced in cisplatin-treated sensitive parental lines but not their cisR1 sub-clones as indicated by the RNA-seq reads determined in this analysis (2-μg/ml cisplatin treatments for 24 hours). (C) Q-RT-PCR confirmed and expanded upon the differential expression of ATF3 in cisplatin-treated Calu6 cells compared to their Calu6cisR1-3 cisplatin-resistant sub-clones following the cytotoxic 4-μg/ml treatments of cisplatin for up to 24 hours. (D) Western blot analysis of ATF3 expression in cisplatin-treated Calu6 and H23 parental cell lines compared to their Calu6cisR1-3 and H23cisR1-3 cisplatin-resistant sub-clones following the cytotoxic 4-μg/ml treatments of cisplatin for 24 hours.


RNA-Seq Transcriptome Analyses
2μg/mL cisplatin, 24 h
A
B
Calu6 vs
Calu6cisR1
932 genes
H23 vs
H23cisR1
601 genes
73 common hits, Log2>+/-2
ATF3 most differentially expressed apoptosis regulator
Calu6
H23
RNA-Seq Reads
untreated
2μg/mL cisplatin
Calu6cisR1
H23cisR1
ATF3
ATF3
ATF3
ATF3
C
D
ATF3 mRNA Levels
Relative Quantitation
Hours (4μg/ml cisplatin treatment)
Calu6
R1
R2
R3
4μg/mL
cisplatin
Calu6
cisR1
cisR2
cisR3
ATF3
actin
H23
cisR1
cisR2
cisR3
ATF3
actin

3.1.3 Cisplatin Induces ATF3 in NSCLC Tumors

ATF3 as a cisplatin-regulated gene has not been previously recognized. To determine whether cisplatin can induce ATF3 expression in a more clinically relevant model, we employed patient-derived surgically excised NSCLC tissue. Tumor tissue and adjacent normal tissue samples were collected from patients undergoing surgery for NSCLC and tissue was treated *ex-vivo* with cisplatin for 48 hours. ATF3 expression was evaluated by RT-qPCR with a 5-fold induction considered as a significant induction that was in line with the cell line data. In the six patients evaluated, cisplatin treatment failed to induce ATF3 expression in all 6 normal lung tissues evaluated while three of the six tumor tissues treated with cisplatin significantly induced ATF3 expression (Figure 7).

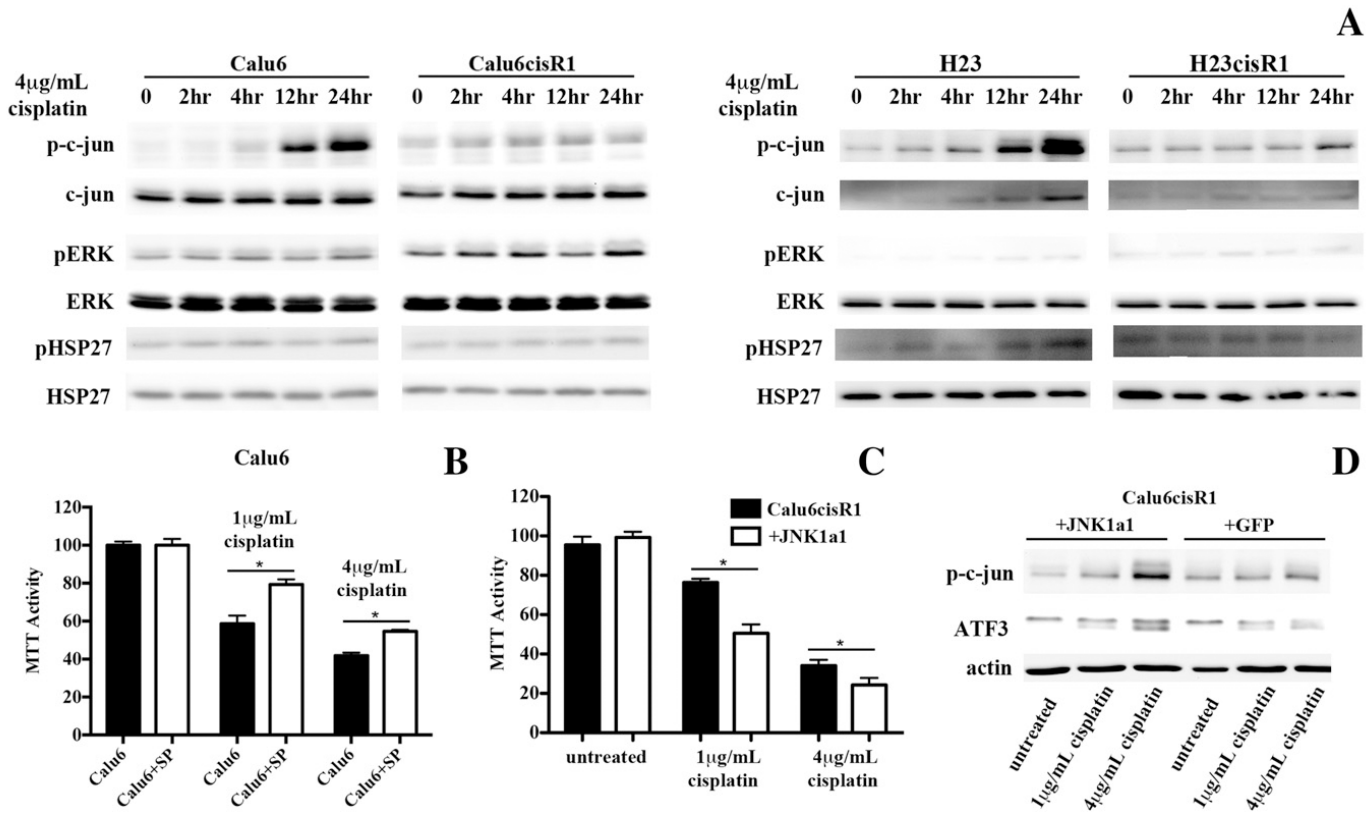
Figure 7: Levels of ATF3 mRNA following solvent or cisplatin treatments (0, 4, and 8 μg/ml) for 48 hours in six NSCLC (T) and adjacent normal (N) ex vivo tissues. Cisplatin-induced expression of greater than five-fold was observed in three of six NSCLC tissue samples evaluated but in none (0/6) of the normal adjacent tissues evaluated.


N01
T01
N02
T02
N03
T03
N04
T04
N05
T05
N06
T06
RQ (ATF3 mRNA)
control
4μg/mL cisplatin
8μg/mL cisplatin

3.1.4 JNK Activation Regulates Cisplatin Induced ATF3 Expression

Previous independent studies have demonstrated the role of MAPKinase pathways in mediating both cisplatin- induced cytotoxicity and ATF3 expression [209, 210, 258]. MAPKinases are involved regulating cellular responses to a diverse array of stimuli and regulate cell functions including cell survival, and apoptosis. To determine the role of the MAPK signaling pathways in regulating ATF3 expression our cisplatin-resistant models, I evaluated the activation of the MAPKinase pathways JNK (assayed through the phosphorylation of its target c-jun [259]), ERK (assayed by p-ERK levels), and p38 (assayed through the phosphorylation of its target HSP27 [260]). Treatment of the Calu6 and H23 parental and cisR1 sub-clones with 4μg/mL cisplatin resulted in a significant time-dependent activation of JNK in the parental cells that was not observed in the cisR1 sub-clones (Figure 8A). Significant activation of ERK or p38 pathways was not observed in these parental or cisR1 cells. Because cisplatin treatment failed to activate JNK in the cisR1 cells, I next evaluated the role of JNK in mediating both cisplatin resistance and their lack of ATF3 induction. I treated the Calu6 cells using a chemical inhibitor of JNK (SP600125) followed by treatment with cisplatin at 1 or 4μg/mL for 48 hours and measured cell viability by MTT assay. Inhibition of JNK resulted in reduced sensitivity to cisplatin treatment (Figure 8B). Conversely, transfection of a JNK activating plasmid in to the Calu6cisR1 cells resulted in enhanced the cisplatin- induced cytotoxicity (Figure 8C). Furthermore, activation of JNK rescued cisplatin induced expression of ATF3 in the Calu6cisR1 (Figure 8D). Together these data demonstrate that ATF3 induction in the NSCLC cell lines is regulated by the activation of JNK, and that dysregulation of JNK leads to impaired ATF3 induction and resistance to cisplatin.

Figure 8: (A) Following 4-μg/ml cisplatin treatments for up to 24 hours, Western blot analysis showed differential induction of JNK MAPKinase activation (through phosphorylated c-jun) but not ERK (phospho-ERK) or p38 (through phosphorylated HSP27) in both Calu6 and H23 cells. This induction was abrogated in their respective cisR1 sublines. (B) MTT analysis of Calu6 cells following 48-hour treatments of (0, 1, and 4 μg/ml) cisplatin with or without 10-μM treatment of the JNK inhibitor SP600125. *Significant differences between columns highlighted ($P < 0.05$). (C) Exogenous expression of a JNK-activated construct enhanced cisplatin cytotoxicity in the Calu6cisR1 subline as assessed by MTT cell viability assay following 48-hour cisplatin treatments. *$P< 0.05$ between columns highlighted. (D) Exogenous expression of activated JNK in Calu6cisR1 also rescued cisplatin-inducible ATF3 expression in these cells assessed by Western blot analysis (24-hour treatments).

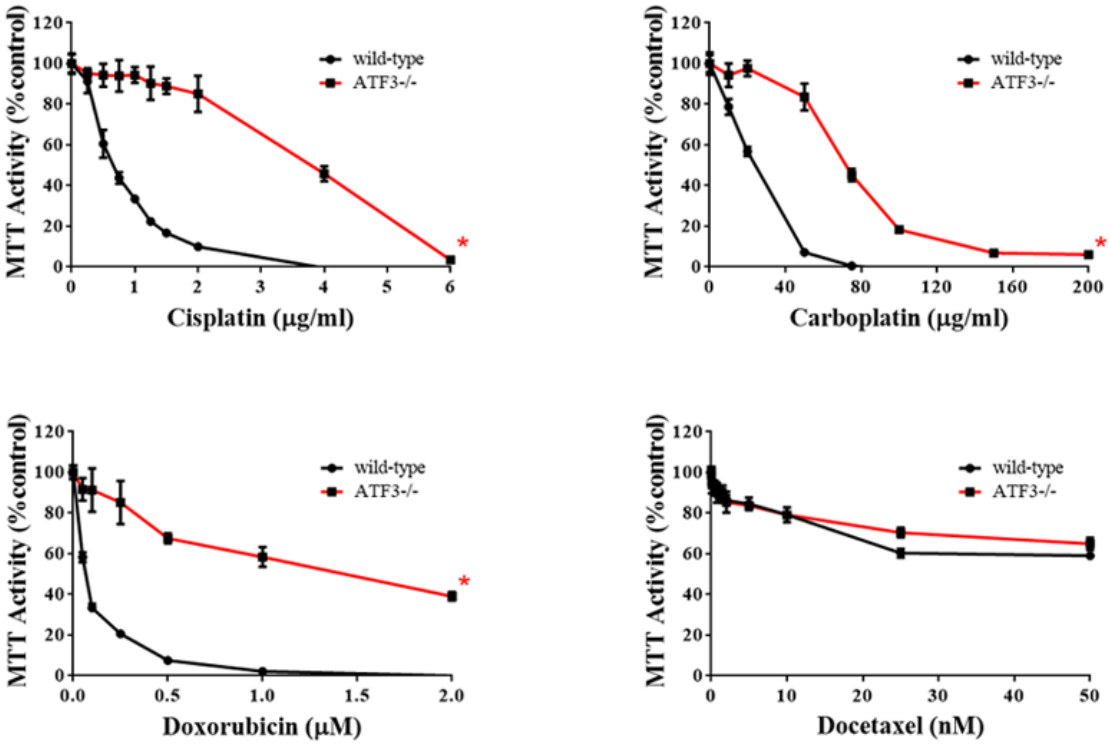

A
Calu6
Calu6cisR1
H23
H23cisR1
4μg/mL cisplatin
0 2hr 4hr 12hr 24hr
p-c-jun
c-jun
pERK
ERK
pHSP27
HSP27
B
Calu6
MTT Activity
1μg/mL cisplatin
4μg/mL cisplatin
Calu6
Calu6+SP
C
Calu6cisR1
+JNK1a1
untreated
1μg/mL cisplatin
4μg/mL cisplatin
D
Calu6cisR1
+JNK1a1
+GFP
ATF3
actin
untreated
1μg/mL cisplatin
4μg/mL cisplatin

3.1.5 ATF3 Expression is Induced by DNA-Damaging Agents

We next evaluated the direct role of ATF3 in regulating the cytotoxicity of a number of chemotherapeutic agents by employing ATF3 null (-/-) cells and their wild-type MEF counterparts. The evaluated chemotherapeutic agents were cisplatin, carboplatin, doxorubicin (DNA damaging agent with a different mechanism of action), and docetaxel (microtubule structure). ATF3 knockout MEFs demonstrated resistance to the DNA-damaging agents tested by MTT cell viability assay (Figure9A). The IC50 values at 48 hours in the WT MEFs compared to the ATF3 knockout MEFs were 0.7 vs 3.7μg/ml) for cisplatin, 21.8 vs 72.8μg/ml for carboplatin, and 0.06 vs. 1.4μM for doxorubicin (Figure 9B). Loss of ATF3 did not significantly affect the sensitivity of the MEFs to docetaxel, which targets tubulin cytoskeleton and acts as a mitotic poison [261]. Similarly, the Calu6 and H23 cell lines and their cisR1 sub-clones demonstrated no difference in sensitivity to docetaxel treatment (Figure 9C). As shown in Figure 1, cisR1 cell lines had reduced sensitivity to treatment with platins but not doxorubicin, nonetheless in the MEFs, doxorubicin induced cytotoxicity appears to be dependent on ATF3 which requires further study. ATF3 expression levels were evaluated by treating the parental and cisR1 cells for 24 hours with the IC50 concentration for each drug, which allowed for the comparison of ATF3 expression with similar levels of cellular toxicity. Treatment with cisplatin and carboplatin readily induced ATF3 expression in the parental cells but not in the cisR1 (Figure 9D). Doxorubicin treatment was able to strongly induce ATF3 expression in both the parental and cisplatin resistant cells while only a weak induction of ATF3 was observed following treatment with docetaxel in the H23 and H23cisR1(Figure 9D).

Figure 9: (A) MTT analysis of wild-type and ATF3−/− MEFs following 48-hour treatments of cisplatin, carboplatin, doxorubicin, and docetaxel. Loss of ATF3 inhibits cisplatin-, carboplatin-, and doxorubicin-induced cytotoxicity but had no effect on docetaxel cytotoxicity. *Significant difference in the viability curves comparing the wild-type and the ATF3−/− MEFs responses (Pb .05). (B) IC50 concentrations for the treatment of the wild-type and knockout MEFs in micromolar (uM) for the indicated drugs. Concentration of cisplatin and carboplatin are also provided as ugl/ml (C) MTT analysis of Calu6, Calu6cisR1, H23, and H23cisR1 following 48-hour treatments with docetaxel showing no significant differences in sensitivity (note: cisplatin, carboplatin, and doxorubicin treatments were presented in Figure 5A). (D) Western blot analysis shows significant ATF3 induction restricted to the parental cell lines with cisplatin and carboplatin treatments. Doxorubicin treatments showed similar ATF3 induction in both the parental and their cisR1 sublines. No significant ATF3 induction was observed with the docetaxel treatments in any of the lines tested, with a weak induction in the H23 series (24-hour treatments, IC50 dose).

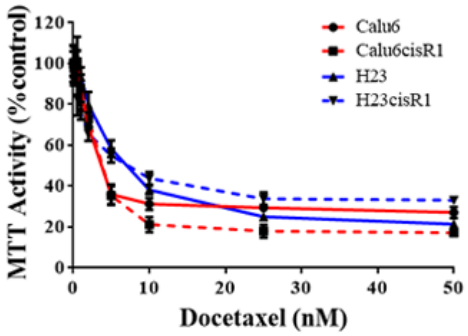
A



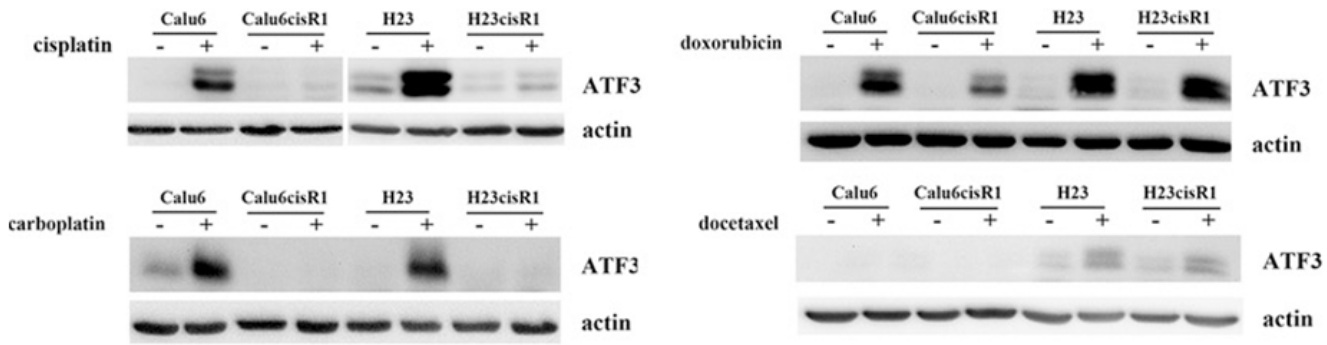
B

MEFs		
	IC50, MTT analysis, 48hr (μM)	
CISPLATIN	Wild-Type	2.29 (0.7 μg/ml)
	ATF3-/-	12.5 (3.7 μg/ml)
CARBOPLATIN	Wild-Type	58.7 (21.8 μg/ml)
	ATF3-/-	196.1 (72.8 μg/ml)
DOXORUBICIN	Wild-Type	0.1
	ATF3-/-	1.4

C



D



3.1.6 Identification of Novel ATF3 inducing Compounds

By restoring ATF3 expression in the Calu6cisR1 cells through JNK activation we could overcome cisplatin-resistance in the Calu6cisR1 cells (Figure 8). This observation suggested that other ATF3 inducing compounds may enhance cisplatin cytotoxicity and reverse resistance to this agent. I performed a high-throughput FDA-approved drug library screen in order to identify drugs with ATF3-dependent cytotoxicity. Wild-type (+/+) and ATF3 knockout (-/-) MEFs were treated with 5μM of the library compound for 48 hours and cell viability was assessed through MTT assay. A "hit" was defined as an agent that displayed greater than 20% cytotoxicity in the ATF3 wild-type MEFs compared to the knockouts. Fifty-seven compounds were identified as "hits", which represented a 3% "hit" rate. The identified compounds belonged to a number of drug classes with varying mechanisms of actions. Table 1 highlights some of the top hits from the screen. Of the compounds identified, we had previously demonstrated three to be ATF3 inducers (Highlighted in red in Table 1) [208, 262, 263]. A second screen was performed using the same library to identify compounds that enhance cisplatin cytotoxicity. Calu6 cells were treated for 24 hours with 1μM of each library compound followed by a 48 hour treatment with 0.1μM cisplatin. This screen identified 12 agents that enhanced cisplatin cytotoxicity by greater than 20% compared to cisplatin or the library compound alone (Table 2).

Table 1: Compounds from library screen demonstrating differential sensitivity in the ATF3 wild-type and knockout MEFs

ATF3+/+ vs. ATF3-/- (5μM Library Compound for 48 hours)

NSAIDs	Topo I Inhibitors	Antimetabolites	Metabolic Stressors	Antibiotics
zomepirac	topotecan	zalcitabine	atovaquone	cefmetazole
piroxicam	etoposide	azaguanine-8	vorinostat	minocycline
	podophyllotoxin	mercaptopurine	monensin	quinacrine
		thioguanosine	fluvastatin	alexidine
		trifluridine	nafronyl oxalate	
			isosorbide	

Table 2: Compounds that enhance cisplatin cytotoxicity identified from 1200 FDA-approved compound library screen

Calu6 (1μM Library Compound + 0.1μg/mL Cisplatin)

Anthracyclins	Topoisomerase I Inhibitor	Microtubule Polymerization	Antimetabolites	Metabolic Stressors
doxorubicin	camptothecin	docetaxel	gemcitabine	vorinostat
daunorubicin	topotecan	nocodazole	floxuridine	antimycin
mitoxantrone			azacytidine	

3.1.7 Inducers of ATF3 Enhance Cisplatin Cytotoxicity

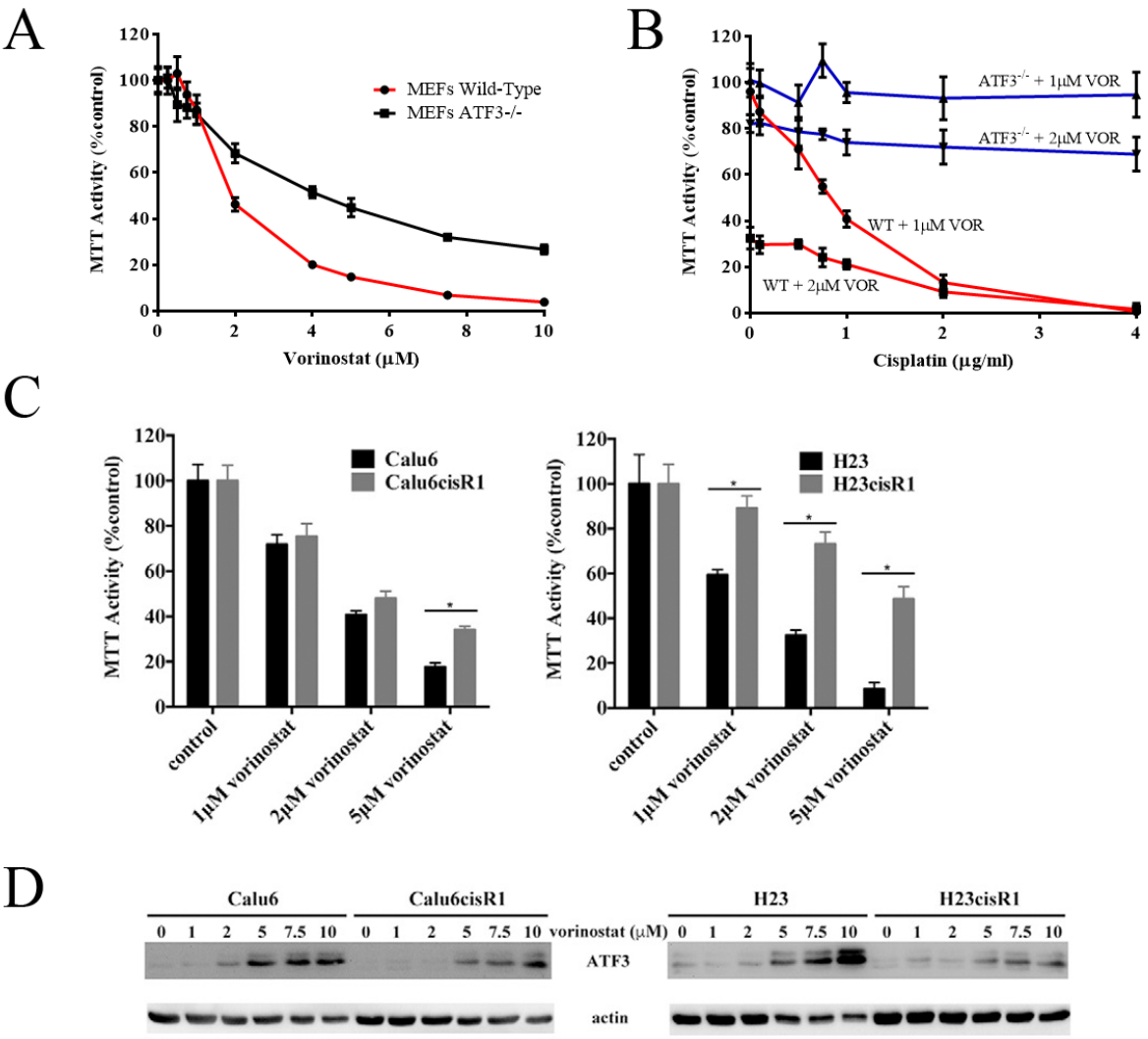
Of interest, vorinostat was one of the compounds identified as both an ATF3 inducer and enhancer of cisplatin cytotoxicity. Previous reports have also shown that vorinostat can enhance cisplatin responsiveness but the cellular mechanisms involved are not known [264]. We first validated the results of the library screen by MTT cell viability assays employing the wild-type and ATF3 knockout MEFs treated with a wide range of vorinostat doses (0-10μM) for 48 hours. ATF3 knockout MEFs demonstrated reduced sensitivity to treatment with vorinostat compared to the wild-type MEFS (Figure 10A). Additionally, 24 hour pre-treatment with vorinostat enhanced cisplatin cytotoxicity in the wild-type MEFs but not in the knockouts; demonstrating that this enhancement in cisplatin cytotoxicity is ATF3-dependent (Figure 10B). In the NSCLC cell lines, the cisR1 sub-clones demonstrated resistance to vorinostat treatment, with reduced cytotoxicity in the H23cisR1 and Calu6cisR1 cells when compared to their respective parental cell lines (Figure 10C). ATF3 expression was strongly induced in the Calu6 and H23 cell lines by vorinostat treatment (Figure 10D) with attenuated induction of ATF3 in the cisR1 cells (Figure 10D). In the NSCLC cell lines, pretreatment with vorinostat (0-, 1-, 2-, or 5 μM for 24 hours) followed by 48 hour treatment with cisplatin enhanced tumor cell cytotoxicity. This was seen in both the parental and cisR1 cells (Figure 10E and F). The evaluation of this combination treatment's effect on ATF3 expression was determined by Western blot. In the Calu6cisR1 and H23cisR1 cells, treatment with cisplatin in combination with vorinostat enhanced ATF3 expression (Figure 10G). Combination index was determined for the combination of cisplatin and vorinostat at 1 and 2 μM in the parental and cisR1 cells (Figure 10H). A synergistic CI (<1)

was found for the combination of cisplatin and 2uM vorinostat in the Calu6, Calu6cisR1, and H23cisR1, and for cisplatin and 1μM vorinostat in the H23 cells.

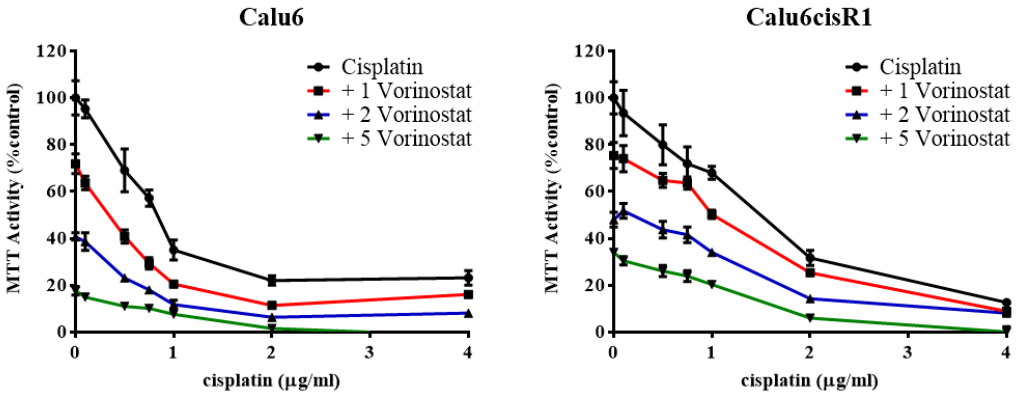
3.1.8 Conclusion

Together the data presented in this study demonstrate that ATF3 plays a key role in mediating the cytotoxic effects of cisplatin in NSCLC. The dysregulation of JNK signaling prevented the induction of ATF3 expression from cisplatin treatment in the cisplatin- resistant NSCLC cell lines and was identified as an important mechanism for cisplatin resistance in our models. We also demonstrated that the combination of ATF3 inducing agents with cisplatin enhanced cisplatin cytotoxicity in the resistant cell lines. Of interest in this study, doxorubicin treatments induced ATF3 expression in both the NSCLC parental and their derived cisplatin-resistant cells and that both the parental and cisplatin-resistant sub-clones displayed similar sensitivity to doxorubicin treatment. Since doxorubicin is also a potent inducer of DNA-damage it was of interest to understand the role of ATF3 in mediating its cytotoxic effect and the cellular mechanisms involved in its upregulation of ATF3 expression as well. Presented in Chapter 4 is our investigation of the role of ATF3 in mediating doxorubicin cytotoxicity in breast cancer models where this agent is clinically relevant.

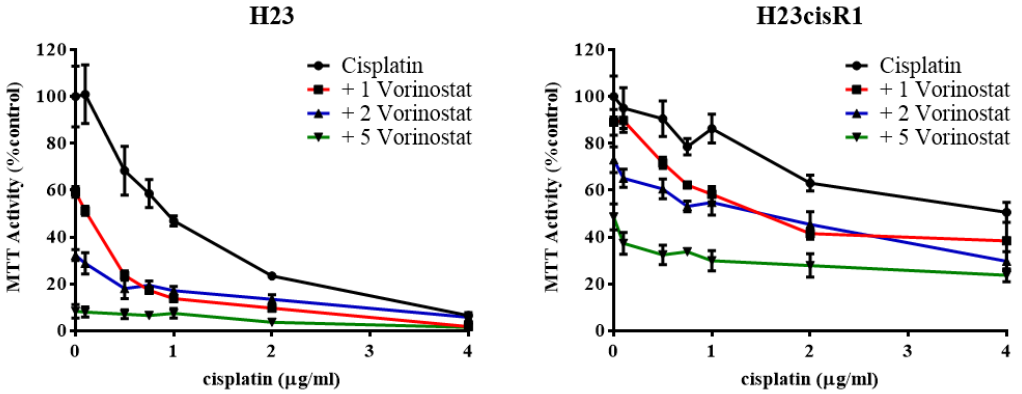
Figure 10: (A) MTT analysis of wild-type and ATF3−/− MEFs following 48-hour treatments with vorinostat showing that loss of ATF3 inhibits vorinostat-induced cytotoxicity in MEFs. (B), MTT analysis of wild-type (wt) and ATF3−/− deficient MEFs following 24-hour pre-treatments of 1 and 2 μM vorinostat followed by cisplatin treatment in combination for a further 48 hours. Enhanced cytotoxicity with the combination was observed only in the wild-type MEFs. (C) MTT analysis comparing response of Calu6 to Calu6cisR1 and H23 to H23cisR1 following 0-, 1-, 2-, and 5-μM vorinostat treatment for 72 hours. The H23cisR1 showed significant resistance to vorinostat. *Significant differences between columns highlighted (Pb .05). (D) Western blot analysis shows ATF3 induced by vorinostat treatments (0-10 μM, 48 hours) in the Calu6 and the H23 parental lines that was evident but attenuated in their cisR1 sub-clones. (E) MTT analysis of Calu6 and Calu6cisR1 and (F) H23 and H23cisR1 following 24-hour pretreatments of 0, 1, 5, and 5 μM vorinostat followed by cisplatin treatment in combination for a further 48 hours. Enhanced cytotoxicity with the combination was observed in all four lines compared to either agent alone. (G) Western blot analysis shows ATF3 induced by 2- and 4-mg/ml cisplatin treatment (24 hours) was enhanced by vorinostat treatment (5 μM, 24-hour pretreatment) in the Calu6cisR1 and the H23cisR1 resistant sublines. (H) CI plots for the combination of cisplatin with vorinostat in the Calu6, Calu6cisR1, H23, and H23cisR1 cells. Cisplatin concentrations used are 1: 0.1ug/ml, 2: 0.5ug/ml, 3: 0.75ug/ml, 4: 1ug/ml, 5: 2ug/ml, and 6: 4ug/ml.


A
MTT Activity (%control)
Vorinostat (μM)
MEFs Wild-Type
MEFs ATF3-/-
B
MTT Activity (%control)
Cisplatin (μg/ml)
ATF3-/- + 1μM VOR
ATF3-/- + 2μM VOR
WT + 1μM VOR
WT + 2μM VOR
C
MTT Activity (%control)
Calu6
Calu6cisR1
control
1μM vorinostat
2μM vorinostat
5μM vorinostat
H23
H23cisR1
D
Calu6
Calu6cisR1
H23
H23cisR1
0 1 2 5 7.5 10 vorinostat (μM)
ATF3
actin

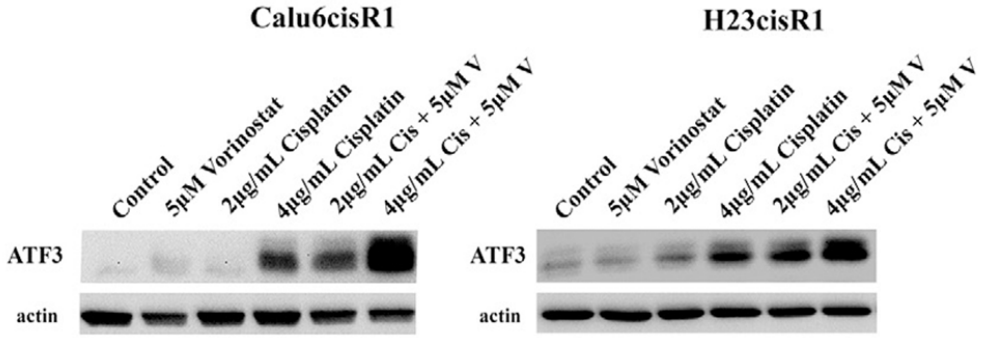
E


Calu6
Calu6cisR1
Cisplatin
+ 1 Vorinostat
+ 2 Vorinostat
+ 5 Vorinostat
MTT Activity (%control)
cisplatin (µg/ml)

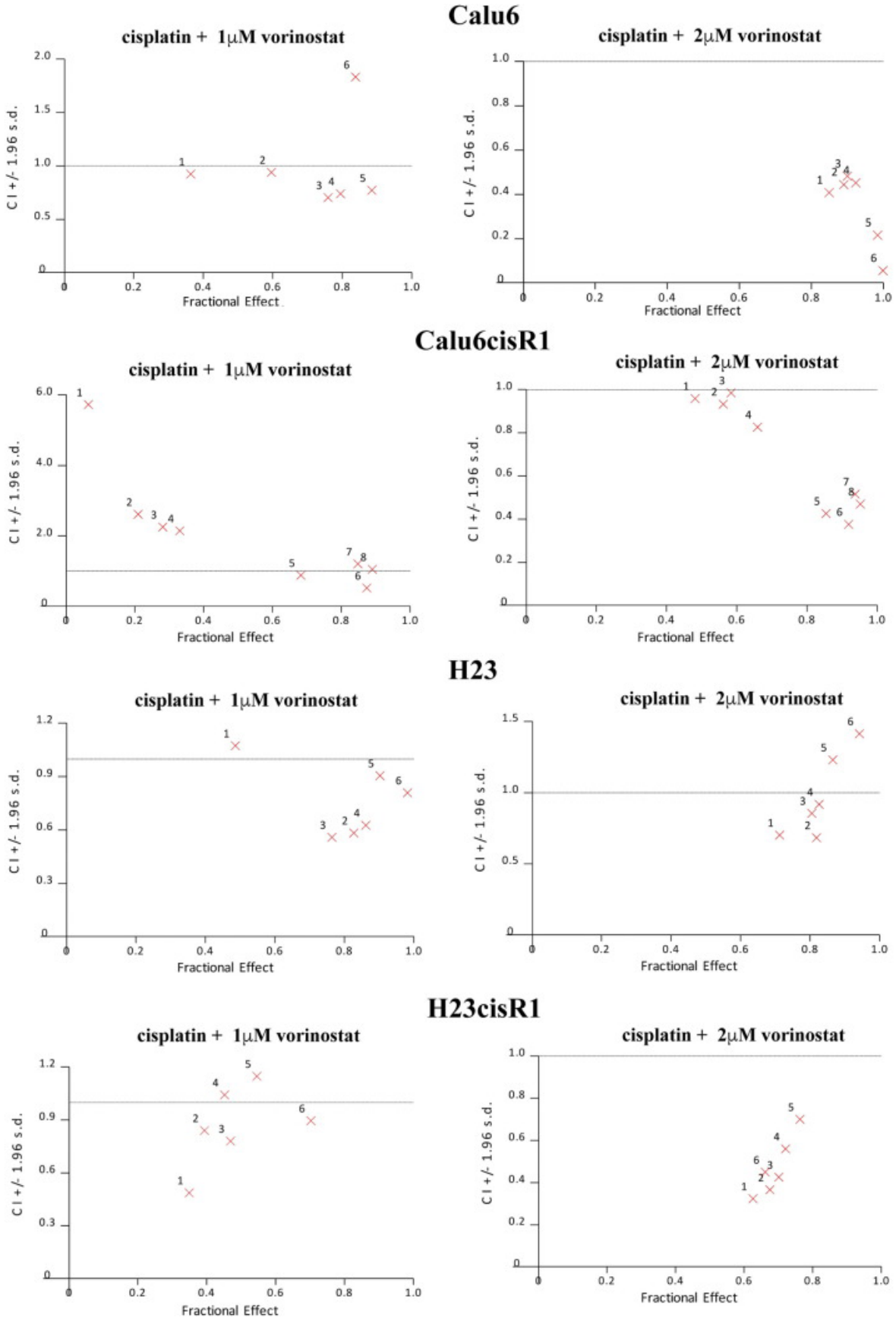
F


H23
H23cisR1
Cisplatin
+ 1 Vorinostat
+ 2 Vorinostat
+ 5 Vorinostat
MTT Activity (%control)
cisplatin (µg/ml)

G


Calu6cisR1
H23cisR1
Control
5µM Vorinostat
2µg/mL Cisplatin
4µg/mL Cisplatin
2µg/mL Cis + 5µM V
4µg/mL Cis + 5µM V
ATF3
actin

H


Calu6
cisplatin + 1μM vorinostat
cisplatin + 2μM vorinostat
Calu6cisR1
cisplatin + 1μM vorinostat
cisplatin + 2μM vorinostat
H23
cisplatin + 1μM vorinostat
cisplatin + 2μM vorinostat
H23cisR1
cisplatin + 1μM vorinostat
cisplatin + 2μM vorinostat
CI +/- 1.96 s.d.
Fractional Effect

Chapter 4: Results

4.1 ATF3 as a Novel Regulator of Chemotherapy Response in Breast Cancer

4.1.1 Doxorubicin Induces ATF3 Expression in Breast Cancer

The role of ATF3 as a potential mediator of doxorubicin's cytotoxicity is not well defined. In this study, we first investigated the ability of doxorubicin to induce ATF3 expression in human breast cancer cell lines representing two common molecular subtypes based on receptor status of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The luminal breast cancer cell line MCF7 (ER+ PR+ Her2-) and the triple-negative cell line MDA-MB-231 (ER- PR- Her2-) were employed in this study. Luminal breast cancers account for approximately 70% of all invasive breast cancers and are considered to be responsive to treatment with chemotherapy [265]. Triple-negative breast cancers account for approximately 15% of invasive breast tumors and often have varying response to treatment with chemotherapy, including doxorubicin [265-267]. The sensitivity of these cell lines to doxorubicin treatment was determined by MTT cell viability assay (Figure 11A). The triple-negative MDA-MB-231 cells were less sensitive (IC50 of; MCF7= 121.4nM; and MDA-MB-231= 287.8nM) to doxorubicin as expected.

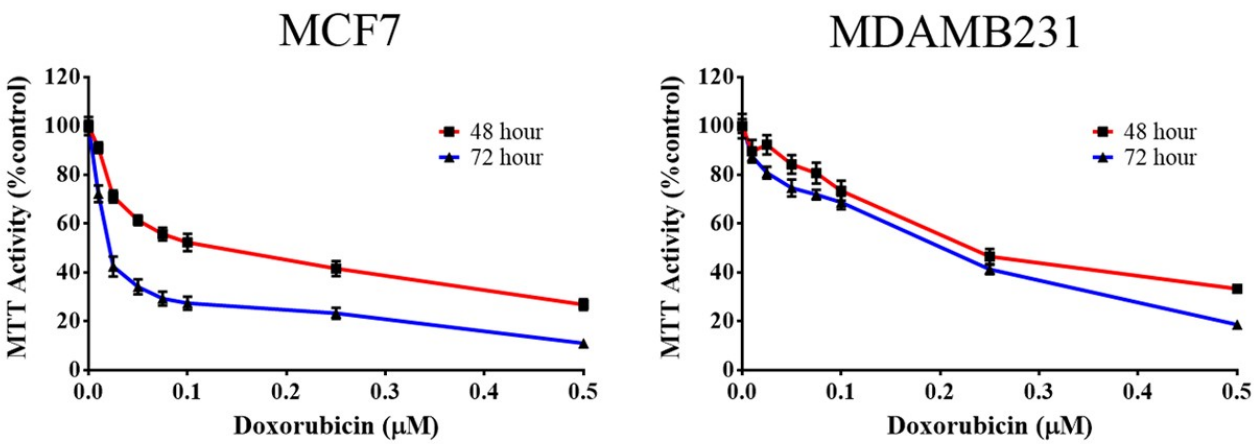
I next evaluated the ability of doxorubicin to induce ATF3 expression employing low (0.1 and 0.25 μM) and high cytotoxic doses of doxorubicin (1μM). Treatment with low dose doxorubicin for 48 and 72 and high dose doxorubicin for 24 hours showed induced ATF3 expression (Figure

11B-D) that was accompanied by an increase in the levels of cleaved-PARP, a marker of cellular apoptosis (Figure 11B). Doxorubicin induced ATF3 expression occurred in a time and dose - dependent manner and was preceded by the accumulation of double strand DNA breaks, as measured by the expression of γH2AX (Figure 11C). Maximal expression of γH2AX was observed at 8 hours in the MDA-MB-231 and 12 hours in the MCF7 cells while maximal ATF3 expression was observed at 24 hours for both cell lines (Figure 11C). RT-qPCR demonstrated that high dose doxorubicin treatment upregulates transcription of ATF3, and its downstream apoptotic effector, DDIT3, a well-established transcriptional target of ATF3 (Figure 11D and E) [268].

These findings suggest that doxorubicin induced DNA damage results in the activation of a DNA-damage response associated with the upregulation of ATF3. I assessed surgically-excised patient tumor samples *ex-vivo* to evaluate ATF3 induction by doxorubicin in a more clinically relevant model. Tumor samples from patients undergoing surgery for breast or ovarian cancer were evaluated. Tissue was processed and treated with doxorubicin for 48 hours upon which RNA was extracted and RT-qPCR was performed to determine ATF3 expression. Three breast and two ovarian tumor samples were tested. In 2/3 breast and 1/2 ovarian tumors, we observed an induction of ATF3 expression of over 5-fold (Figure 11F) compared to untreated tissues. Since ATF3 expression is typically low and in some cases not detectable, to ensure a significant induction, a 5-fold induction was set as a cut-off for significant induction of ATF3. This is the first report that doxorubicin can induce ATF3 expression directly in human tumor tissue.

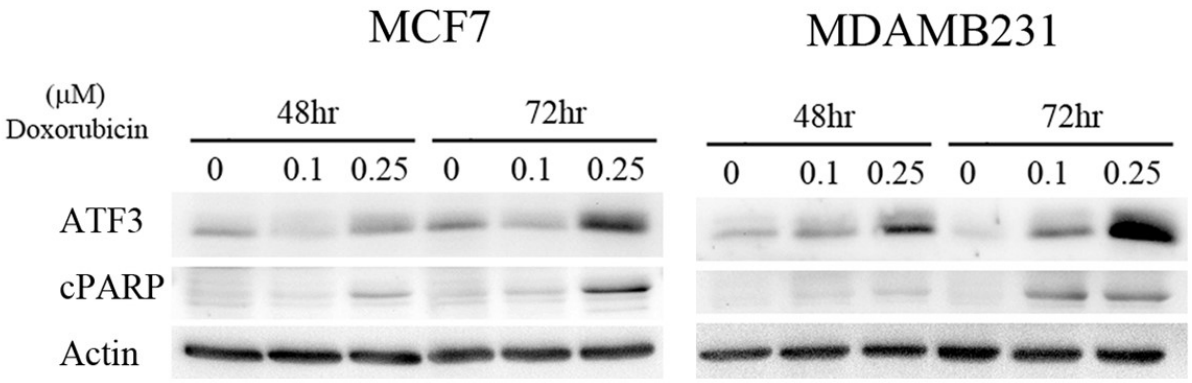
Figure 11: A) MTT analysis demonstrating the sensitivity of the human breast cancer cell lines MCF7 and MDA-MB-231 to doxorubicin following 48 and 72 hour treatment. IC50 concentrations at 48 hours following treatment are provided for each cell line in nanomolar concentrations. Error bars, SD from the mean of three replicates (n = 3) B) Western blots for ATF3 expression in the breast cancer cell lines following 48 and 72 hour treatment with doxorubicin. Upregulation of the expression of cleaved-PARP was observed to occur alongside the induction of ATF3 expression by doxorubicin treatment. C) Western blots demonstrating the upregulation of ATF3 by doxorubicin treatment over a 24 hour time course in all three cell lines. Doxorubicin induced DNA double strand breaks was assessed through the expression of γH2AX. D) and E) RT-qPCR for ATF3 and DDIT3 expression following 24 hour treatment with doxorubicin. Statistical analysis was performed by One-way ANOVA using the Bonferroni multiple comparison test. **: $p \leq 0.01$, *** $p \leq 0.001$ F) RT-qPCR for ATF3 expression in three human breast and two ovarian tumors treated with doxorubicin for 48 hours. Doxorubicin induced ATF3 expression of greater than five-fold was observed in 2/3 breast and 1/2 ovarian tumors evaluated.

A

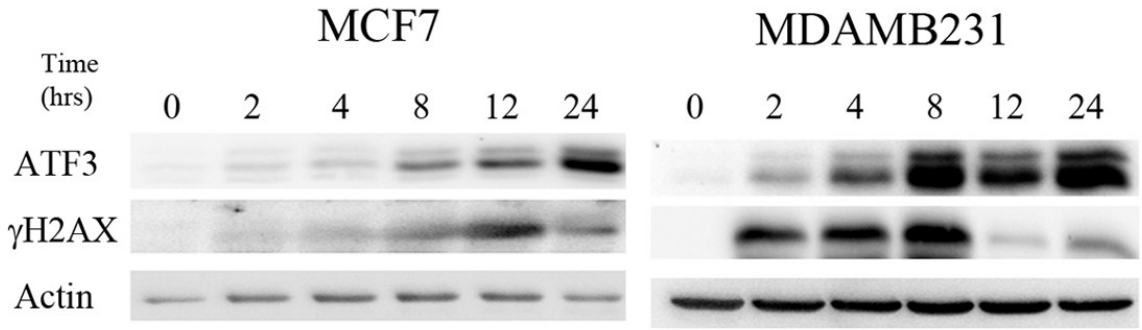


IC50, MTT analysis, 48hr (nM)	MCF7	MDAMB231
	121.4	287.8

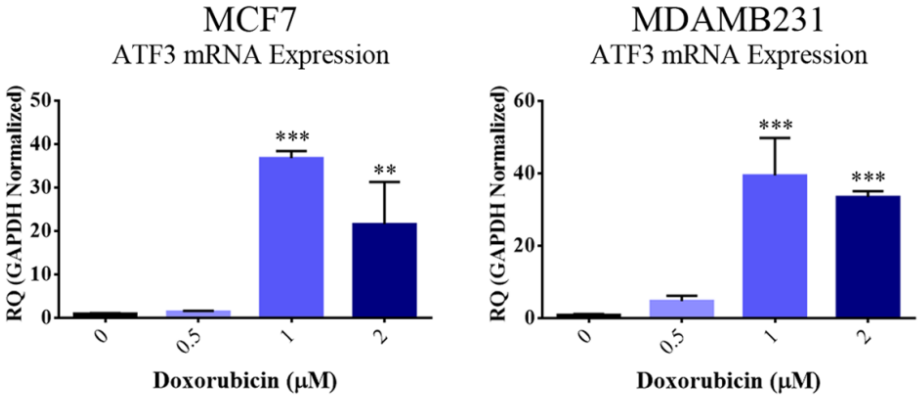
B



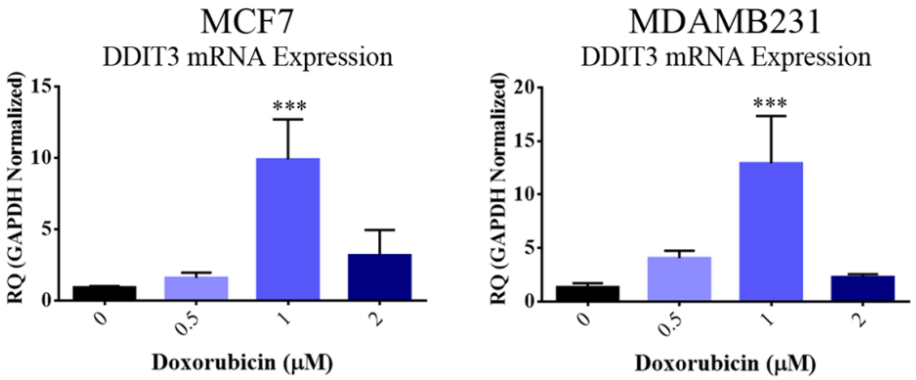
C



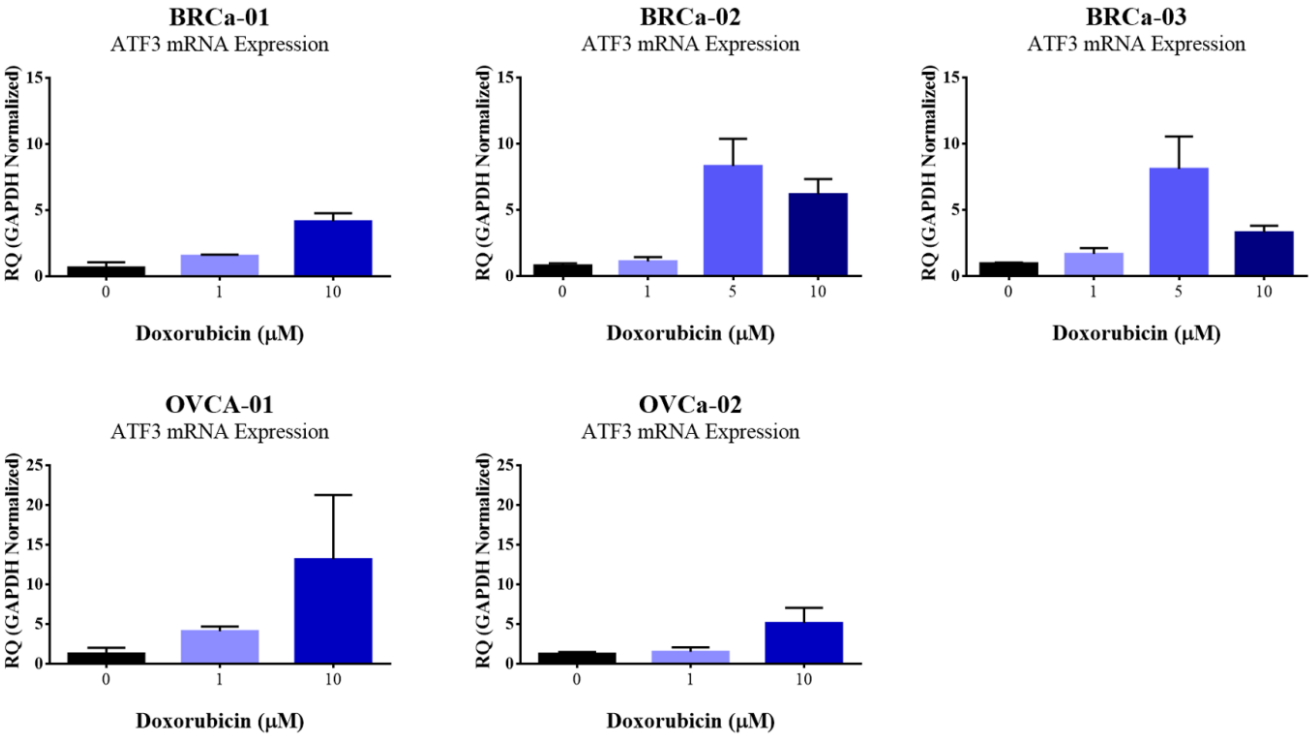
D


MCF7
ATF3 mRNA Expression
RQ (GAPDH Normalized)
***
**
Doxorubicin (μM)
MDAMB231
ATF3 mRNA Expression
RQ (GAPDH Normalized)
***
***
Doxorubicin (μM)

E


MCF7
DDIT3 mRNA Expression
RQ (GAPDH Normalized)
***
Doxorubicin (μM)
MDAMB231
DDIT3 mRNA Expression
RQ (GAPDH Normalized)
***
Doxorubicin (μM)

F

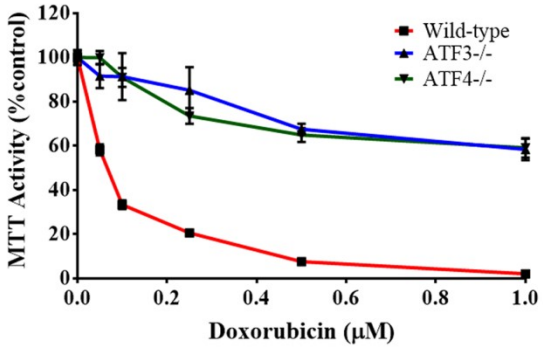

BRCa-01
ATF3 mRNA Expression
RQ (GAPDH Normalized)
Doxorubicin (μM)
BRCa-02
ATF3 mRNA Expression
RQ (GAPDH Normalized)
Doxorubicin (μM)
BRCa-03
ATF3 mRNA Expression
RQ (GAPDH Normalized)
Doxorubicin (μM)
OVCA-01
ATF3 mRNA Expression
RQ (GAPDH Normalized)
Doxorubicin (μM)
OVCa-02
ATF3 mRNA Expression
RQ (GAPDH Normalized)
Doxorubicin (μM)

4.1.2 Doxorubicin Cytotoxicity is Regulated by ATF3

Elevated and sustained levels of ATF3 expression can trigger cellular apoptosis [208]. In order to determine the role of ATF3 in mediating doxorubicin cytotoxicity an ATF3 knockout (ATF3-/-) MEF model was employed. Treatment of the wild-type MEFs (ATF3+/+) with doxorubicin resulted in significant cytotoxicity after 48 hours as determined by the MTT cell viability assay (Figure 12A). ATF3 knockout MEFs displayed a significant reduction in sensitivity to doxorubicin treatment, demonstrating an important role for ATF3 in mediating the cytotoxic effect of doxorubicin. Treatment with docetaxel displayed similar levels of cytotoxicity in both wild-type and ATF3 knockout MEFs, which demonstrates a specific role for ATF3 in doxorubicin cytotoxicity and that the loss of ATF3 did not result in an overall impairment in cell death pathways. Treatment of the wild-type MEFs with doxorubicin (0-, 0.5-, 0.75-, 1-, and 2μM) for 24 hour demonstrated a dose dependent increase of ATF3 expression (Figure 12B). Cleavage of PARP was clearly evident following doxorubicin treatment in the wild-type MEFs but not in the ATF3-/- MEFs, indicating elevated apoptosis in wild-type MEFs compared to their ATF3-/- counterparts (Figure 12B). Employing CRISPR/cas9, we targeted the ATF3 gene in the MCF7 cell line. Loss of ATF3 in the MCF7 resulted in a relatively weak reduction in sensitivity to doxorubicin, with a small effect observed at low concentrations of doxorubicin at a longer time point of 72 hours (Figure 12D). The change in IC50 of the parental MCF7 cells versus the ATF3-CRISPR knockout sub-clone was 8.4nM to 19.9nM following 72 hour treatment.

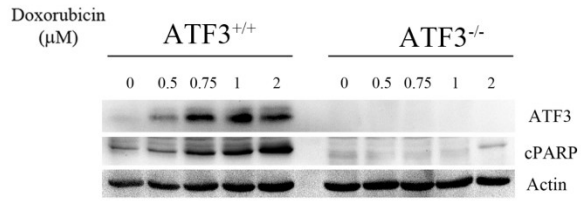
Figure 12: A) MTT analysis in wild-type, ATF3$^{-/-}$, and ATF4$^{-/-}$ MEFs treated with doxorubicin for 48 hours. IC50 concentrations at 48 hours following treatment are provided for each cell line in micromolar concentrations. As observed, loss of ATF3 resulted in a significant reduction in sensitivity to doxorubicin treatment. Error bars, SD from the mean of three replicates (n = 3) B) Western blot analysis for wild-type and ATF3$^{-/-}$ MEFs treated with various concentrations of doxorubicin for 24 hours. C) Western blot analysis for wild-type and ATF4$^{-/-}$ MEFs treated with various concentrations of doxorubicin for 24 hours. D) MTT analysis is MCF7 and MCF7 ATF3 CRISPR knockout cell treated with doxorubicin for 72 hours with Western blot analysis for ATF3 expression. IC50 concentrations at 72 hours following treatment are provided for each cell line in nanomolar concentrations.

A

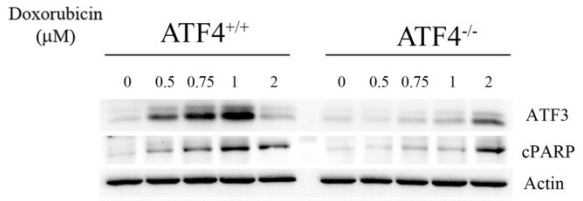


IC50, MTT analysis, 48hr (μM)	
Wild-Type	0.07
ATF3-/-	1.4
ATF4-/-	1.3

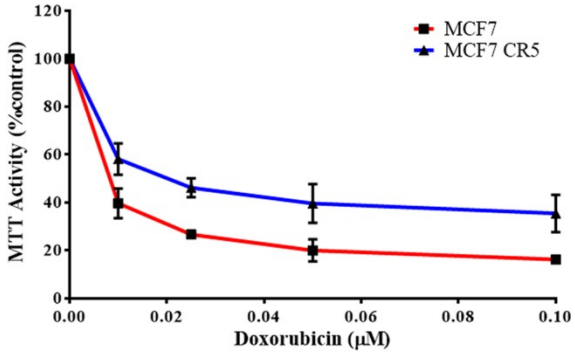
B



C

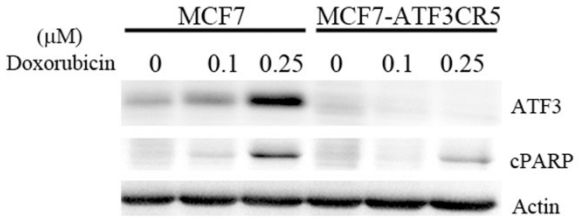


D



IC50, MTT analysis, 72hr (nM)	
MCF7	8.4
MCF7CR5	19.9

E



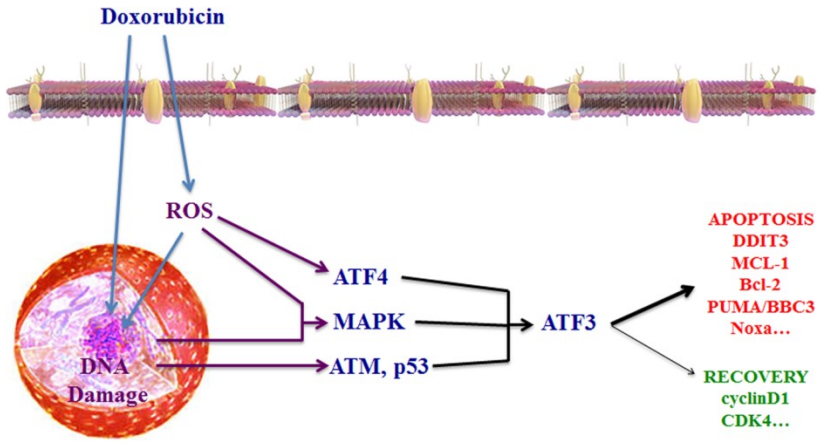
4.1.3 The ISR, DDR, and MAPK Signaling Pathway Regulate Doxorubicin Induced ATF3 Expression

Understanding the molecular mechanisms regulating ATF3 expression induced by doxorubicin may uncover novel therapeutic targets. Doxorubicin has been demonstrated to activate various cellular signaling pathways and processes including ISR, DDR and MAPK signaling pathways [269-271]. Additionally, upregulation of ATF3 has been demonstrated to occur following a variety of cellular stresses which activate various signaling pathways including those triggered by doxorubicin treatment. For example, the upregulation of ATF3 occurs through the activation of the ISR, DDR, and MAPK signaling pathways (Figure 9A) [208, 210, 272]. The apoptotic role of ATF3 has been best characterized as part of the ISR. During the ISR, a variety of cellular stressors result in the phosphorylation of eIF2α which inhibits global protein translation. A subset of mRNAs, including ATF4 and ATF3, that contain upstream open reading frames are still able to be translated resulting in their upregulation. Employing ATF4 knockout (-/-) MEFs and their paired wild-type counterpart I demonstrated that the loss of ATF4 expression also results in a significant reduction in sensitivity to doxorubicin treatment, similar to what was observed in the ATF3 knockout MEFs (Figure 12A). Loss of ATF4 expression also resulted in an attenuation of doxorubicin induced ATF3 expression and cleaved-PARP following 24 hour treatment (Figure 12C). Doxorubicin is a known potent inducer of reactive oxygen species (ROS) and able to initiate the ISR through endoplasmic reticulum stress [269]. Pre-treatment of the MCF7 breast cancer cells with n-acetylcysteine (NAC), a commonly used ROS scavenger , resulted in an attenuation of doxorubicin induced ATF3 expression (Figure 13B). These data suggest that doxorubicin, in part, induces ATF3 through the activation of both ROS and the ISR.

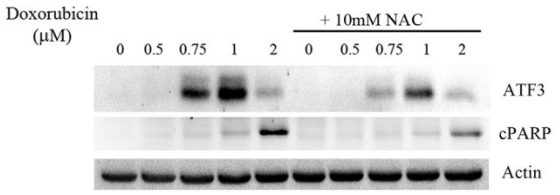
Since doxorubicin is primarily considered to be a DNA-damaging agent, I investigated the potential role of cellular mechanisms involved in the cellular response to DNA-damage. Previously, we demonstrated that the MAPK signaling pathway is responsible for mediating cisplatin induced ATF3 expression. MCF7 cells were pre-treated with chemical inhibitors for JNK (SP600125), ERK1/2 (U0126), or p38 (SB203580) followed by doxorubicin treatment and ATF3 expression was assessed by Western blot. Inhibition of JNK resulted in an attenuation of doxorubicin induced ATF3 expression (Figure 13C). ERK1/2 (Figure 13E) and p38 (Figure 13D) inhibition did not result in a reduction in doxorubicin induced ATF3 expression. Therefore, JNK signaling plays a role in mediating doxorubicin induced ATF3 expression. ATM is a kinase that is activated in response to DNA double-strand breaks [273]. Inhibition of ATM with the inhibitor KU55933 resulted in reduced ATF3 expression following doxorubicin treatment (Figure 13F). Together these results demonstrate the role of three major signaling pathways in regulating ATF3 expression induced by doxorubicin in MCF7 cells.

Figure 13: A) Schematic depicting the potential mechanisms involved in regulating doxorubicin induced ATF3 expression. DNA-damage and ROS produced by doxorubicin treatment can initiate various signaling pathways, such as the ISR through ATF4, the MAPK signaling pathways, and DDR pathways. Upregulation of ATF3 by these signaling pathways can lead to both cell recovery and apoptosis. Cell death occurs when ATF3 expression is elevated and sustained. B-F) Western blot analysis for ATF3 expression following 24 hour doxorubicin treatment with or without n-acetylcysteine (NAC) or chemical inhibitors for JNK (SP600125), ERK1/2 (U0126), p38 (SB203580), and ATM (KU55933) in the MCF7 cells

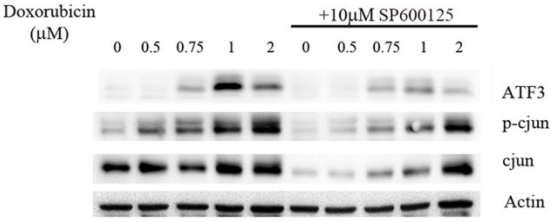
A


Doxorubicin
ROS
ATF4
MAPK
ATM, p53
ATF3
DNA Damage
APOPTOSIS
DDIT3
MCL-1
Bcl-2
PUMA/BBC3
Noxa…
RECOVERY
cyclinD1
CDK4…

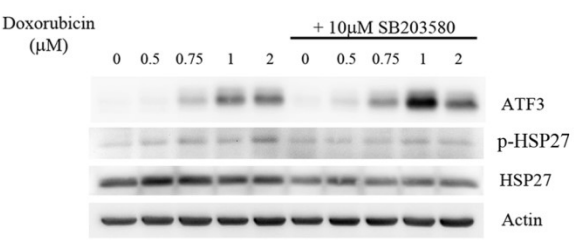
B


Doxorubicin (μM)
+ 10mM NAC
0 0.5 0.75 1 2 0 0.5 0.75 1 2
ATF3
cPARP
Actin

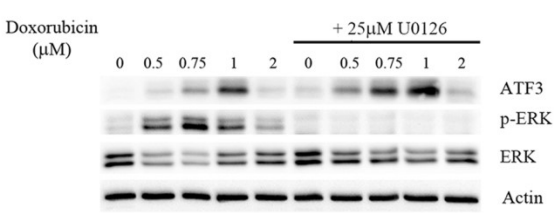
C


Doxorubicin (μM)
+10μM SP600125
0 0.5 0.75 1 2 0 0.5 0.75 1 2
ATF3
p-cjun
cjun
Actin

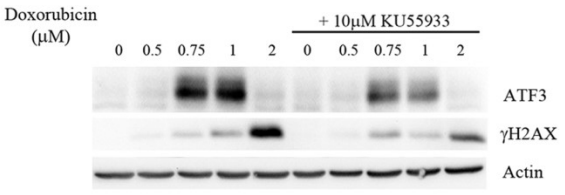
D


Doxorubicin (μM)
+ 10μM SB203580
0 0.5 0.75 1 2 0 0.5 0.75 1 2
ATF3
p-HSP27
HSP27
Actin

E


Doxorubicin (μM)
+ 25μM U0126
0 0.5 0.75 1 2 0 0.5 0.75 1 2
ATF3
p-ERK
ERK
Actin

F

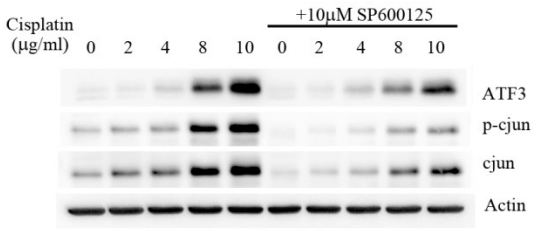

Doxorubicin (μM)
+ 10μM KU55933
0 0.5 0.75 1 2 0 0.5 0.75 1 2
ATF3
γH2AX
Actin

4.1.4 Cisplatin Induces ATF3 Expression in Breast Cancer through MAPK Signaling Pathway

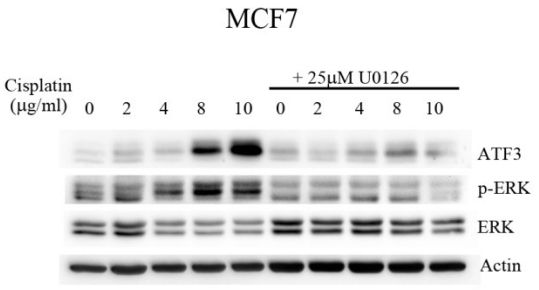
I subsequently investigated the ability of cisplatin to induce ATF3 expression in the MCF7 breast cancer cell line as a comparator DNA damaging agent with a different mechanism that induces ATF3 in NSCLC cell lines as demonstrated above. Cisplatin treatment for 24 hours (0-, 2-, 4-, 8-, and 10μg/mL) resulted in a dose dependent increase in ATF3 expression (Figure 14A-C). Previously, we demonstrated that MAPK signaling played an important role in mediating ATF3 induction following cisplatin treatment. In the MCF7 cell lines, inhibition of JNK (Figure 14A), ERK1/2 (Figure 14B), and p38 (Figure 14C) all resulted in an attenuation of ATF3 expression following cisplatin treatment. These data further demonstrate the role of MAPK signaling pathways in regulating ATF3 expression and suggests the MAPK signaling pathways involved are context and drug dependant.

Figure 14: A-C) Western blot analysis for ATF3 expression following 24 hour cisplatin treatment with chemical inhibitors for JNK (SP600125), ERK1/2 (U0126), p38 (SB203580), and ATM (KU55933) in the MCF7 cells

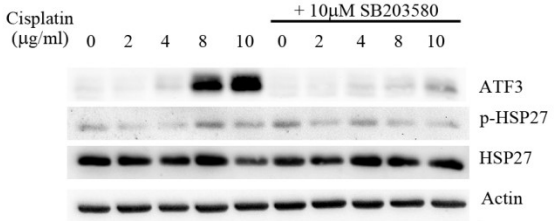
A


MCF7
Cisplatin (μg/ml)
0 2 4 8 10
+10μM SP600125
0 2 4 8 10
ATF3
p-cjun
cjun
Actin

B


MCF7
Cisplatin (μg/ml)
0 2 4 8 10
+ 25μM U0126
0 2 4 8 10
ATF3
p-ERK
ERK
Actin

C


MCF7
Cisplatin (μg/ml)
0 2 4 8 10
+ 10μM SB203580
0 2 4 8 10
ATF3
p-HSP27
HSP27
Actin

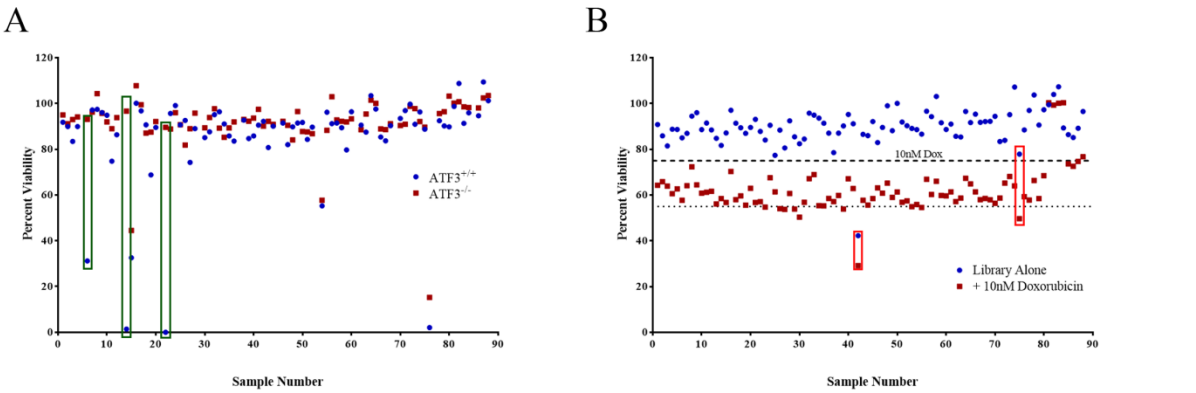
4.1.5 Identification of ATF3 Inducing Compounds that Enhance Doxorubicin Cytotoxicity

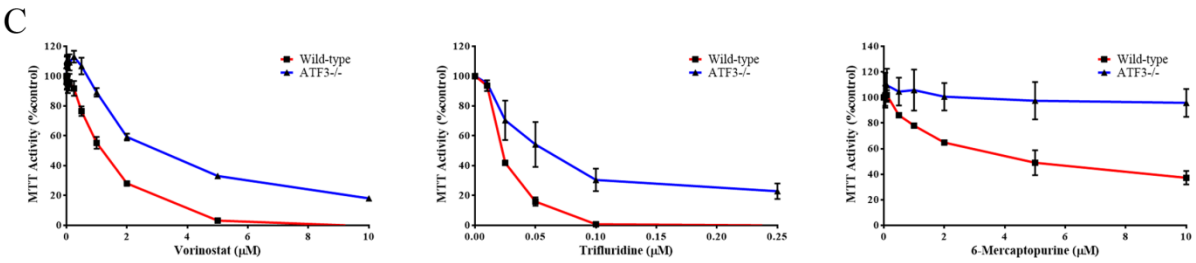
The identification of compounds that can enhance the efficacy of doxorubicin will allow for novel combination strategies that will enhance the efficacy or mitigate the severe toxicities of doxorubicin treatments by allowing for lower doses without effecting efficacy. The combination of ATF3 inducing compounds with doxorubicin may provide a means to sustain and elevate ATF3 expression in order to induce tumor cell death and enhance treatment efficacy. To this end, we performed a 1200 compound library screen of FDA approved agents to identify drugs whose cytotoxicity was dependant on ATF3 (Table 1). ATF3+/+ and ATF3-/- MEFs were treated for 48hrs with 5μM of each compound and assayed for changes in cell viability by the MTT assay. Of the 121 drugs that displayed cytotoxicity in the wild-type MEFs, 57 compounds displayed a greater than 20% cytotoxicity in the ATF3+/+ MEFs compared to the ATF3-/- MEFs. A representative plot of the data obtained from the library screen is shown in Figure 15A. While most compounds have minimal or no cytotoxic effect at the concentration tested, highlighted are a few which demonstrated drastic differences in sensitivity to the library compound.

Using the same 1200 compound library, I screened for compounds that enhance doxorubicin cytotoxicity as well. MCF7 cells were pretreated for 24 hours with the library compound followed by 48 hour treatment with 10nM doxorubicin (concentration that is ~20% cytotoxic). The screen identified 10 compounds that enhanced doxorubicin cytotoxicity by greater than 20% compared to the library compound and doxorubicin alone. Our laboratory had previously identified monensin and vorinostat as inducers of ATF3 [212, 262]. Vorinostat, an HDAC

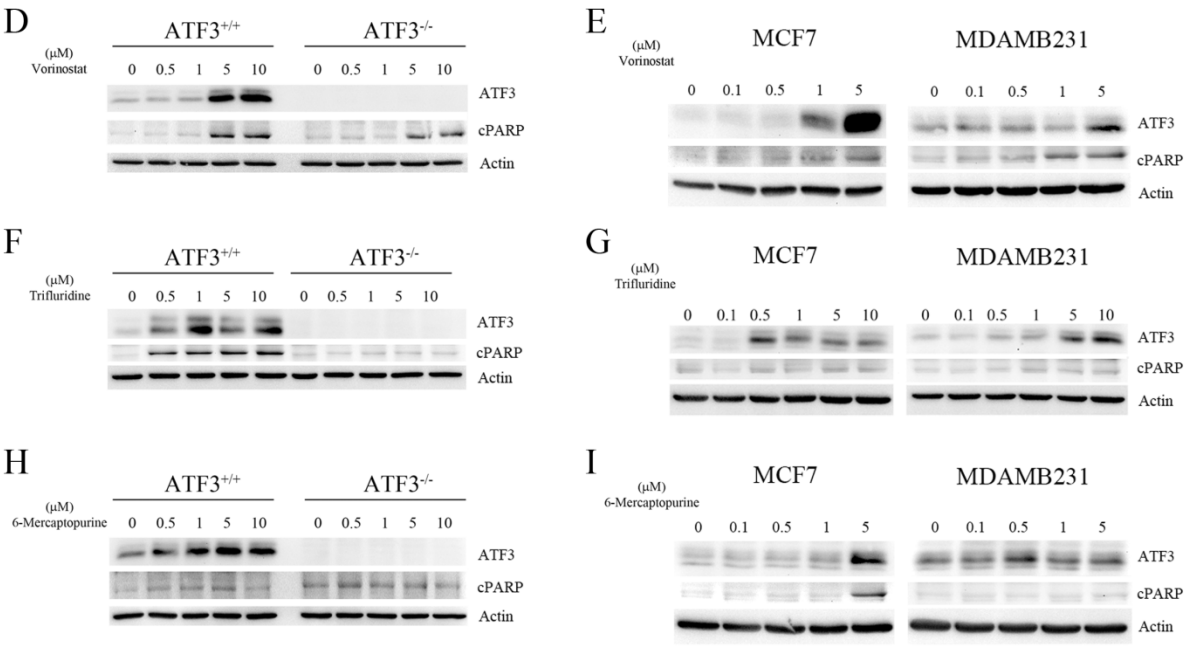
inhibitor, and the two nucleoside analogues, trifluridine and 6-mercaptopurine, are currently being employed as chemotherapeutic agents and were chosen for further evaluation. Validation of the screen was performed by treating the ATF3+/+ and ATF3-/- MEFs with a dose curve of each compound for 48 hours and assessing cell viability through MTT assay. For the three compounds tested loss of ATF3 resulted in a reduction in sensitivity to drug treatment (Figure 15C). The IC50 values at 48 hours in the WT MEFs compared to the ATF3 knockout MEFs were 1.0 vs. 3.3μM for vorinostat and 0.02 vs. 0.08μM for trifluridine. The IC50 for the WT MEFs for 6-mercaptopurine was 4.8μM and was greater than 10μM in the ATF3 knockout MEFs as less than 50% cell viability was not observed. Western blot analysis demonstrated that vorinostat (Figure 15D and E), trifluridine (Figure 15 F and G), and 6-mercaptopurine (Figure 15 H and I) all induced ATF3 expression in the wild-type MEFs and the MCF7 and MB-231 breast cancer cell lines.

Figure 15: A 1200 FDA approved library screen was performed to identify inducers of ATF3. A) Representative plot of the data obtained from the screen performed in the wild-type and $ATF3^{-/-}$ MEFs. While there was no difference in cytotoxicity for many of the compounds between the two groups, there was a significant reduction in sensitivity to some drugs (highlighted in green). B) Representative plot of the data obtained from the library screen performed in the MCF7 cell lines to identify enhancers of doxorubicin cytotoxicity. C) MTT analysis demonstrating that the loss of ATF3 in MEFs results in a reduction in sensitivity to vorinostat, trifluridine, and 6-mercaptopurine. Error bars, SD from the mean of three replicates (n = 3) D-I) Western blots analysis demonstrating the ability of vorinostat (D-E), trifluridine (F-G), and 6-mercaptopurine (H-I) to induce ATF3 expression in the wild-type MEFs and the human breast cancer cell lines following 24 hour treatment.





MEFs								
	IC50, MTT analysis, 48hr (μM)							
Vorinostat	Wild-Type	1.03	**Trifluridine**	Wild-Type	0.02	**6-Mercaptopurine**	Wild-Type	4.84
	ATF3-/-	3.3		ATF3-/-	0.06		ATF3-/-	N/A



4.1.6 ATF3 Inducing Compounds Enhance Doxorubicin Cytotoxicity

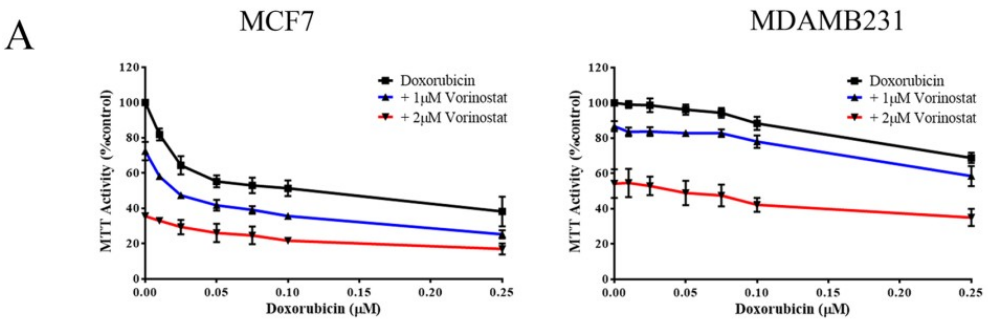
To test whether the identified ATF3 inducers can enhance doxorubicin cytotoxicity, I treated the breast cancer cells for 24 hours with vorinostat (1-, or 2μM) or trifluridine (0.1-, or 0.5μM) followed by 48 hour doxorubicin and assessed cell viability through MTT assay. MCF7 cells displayed enhanced tumor cell cytotoxicity when treated with the combination of vorinostat and doxorubicin (Figure 16A) and the combination treatment of trifluridine and doxorubicin in the MCF7 cells (Figure 16B). Interestingly, vorinostat or trifluridine did not enhance doxorubicin cytotoxicity in the MDAMB231 cells (Figure 16A and B). Following the same treatment course, Western blot analysis demonstrated that the combination of trifluridine and vorinostat can enhance doxorubicin induced ATF3 expression in the MCF7 cells (Figure 16C). In agreement with the MTT data, ATF3 expression was not enhanced by the combination treatment in the MDAMB231 cells (Figure 16C). Lastly, we determined whether the nature of the combination treatment as either additive or synergistic effect through the determination of the combination index. The combination treatment of vorinostat with doxorubicin resulted in an additive effect on cytotoxicity as most of the values from the various combination tested were close to 1 (Figure 16D). A synergistic effect was achieved with the combination of trifluridine and doxorubicin in the MCF7 cells as the combination indexes were consistently below 1(Figure 16E). In the MDAMB231 cells, neither combination resulted in an additive or synergistic effect consistent with the data generated above (Figure 16D and E).

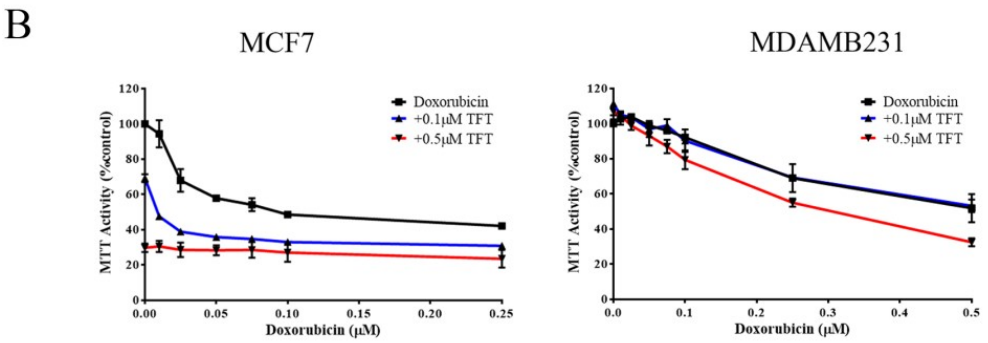
4.1.7 ATF3 Induction by Trifluridine and Vorinostat is Regulated by MAPK Signaling Pathways

In order to determine the potential mechanism involved in mediating the enhancement of doxorubicin cytotoxicity observed with the combination treatments in MCF7 cells, I investigated the role of the MAPK signaling pathway in mediating trifluridine and vorinostat induced ATF3 expression. Similar to doxorubicin treatments, inhibition of JNK but not the other MAPK pathways attenuated ATF3 expression following vorinostat treatment in MCF7 breast cancer cells (Figure 17A-C). Similar to the cisplatin treatment results, inhibition of JNK, ERK1/2 and p38 attenuated trifluridine induced ATF3 expression in the MCF7 breast cancer cells (Figure 17D-F). These data suggest that that vorinostat and trifluridine are inducers of ATF3 expression through the activation of the MAPK signaling pathway, and that their enhanced ATF3 expression in combination with doxorubicin may also enhance the cytotoxicity of these agents in combination. In the MCF7 cells, vorinostat induced ATF3 expression is mediated predominately through the JNK pathway, whereas for trifluridine multiple MAPK signaling pathways are involved. This may explain the additive nature of the vorinostat combination cytotoxicity results with doxorubicin whereas the trifluridine combinations display synergistic cytotoxicity with doxorubicin in MCF7 cells. Further studies are required to elucidate the drivers of these combination cytotoxicity results.

Since doxorubicin use is limited due to significant toxicities, the identification of novel combination strategies provides a means to reduce treatment associated toxicities while maintaining efficacy. The combination of ATF3 inducing compounds with doxorubicin may represent such a combination strategy.

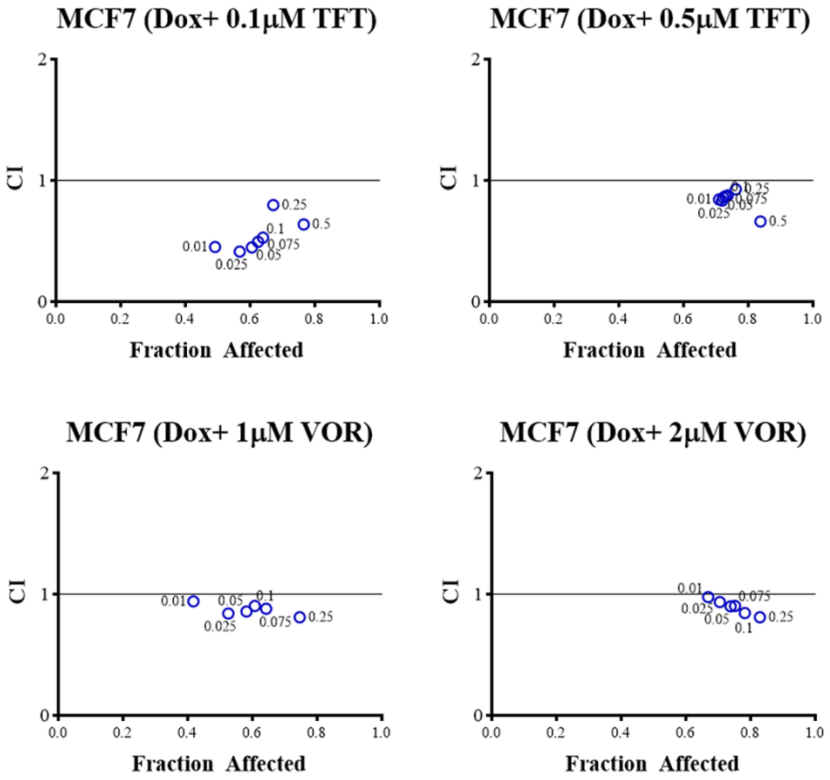
Figure 16: MTT analysis for the combination of vorinostat (A) and trifluridine (B) with doxorubicin in the human breast cancer cell lines. Cells were pretreated with vorinostat or trifluridine for 24 hours followed by 48 hour treatment with doxorubicin. Error bars, SD from the mean of three replicates (n = 3) C) Western blot analysis for the expression of ATF3 following the combination treatment of vorinostat or trifluridine with doxorubicin in the breast cancer cell lines. D) and E) Combination Index (CI) was determined for the combination of vorinostat and trifluridine with doxorubicin in the MCF7 and MDAMB231 cells. CI<1 is synergistic, CI=1is additive, and CI>1 is antagonistic


A
MCF7
MDAMB231
Doxorubicin
+ 1μM Vorinostat
+ 2μM Vorinostat
MTT Activity (%control)
Doxorubicin (μM)


B
MCF7
MDAMB231
Doxorubicin
+0.1μM TFT
+0.5μM TFT
MTT Activity (%control)
Doxorubicin (μM)


C
MCF7
MDAMB231
+ 0.5μM TFT
+ 1μM SAHA
Doxorubicin (μM) 0 0.5 1 0 0.5 1 0 0.5 1
ATF3
cPARP
Actin

D


MCF7 (Dox+ 0.1μM TFT)
MCF7 (Dox+ 0.5μM TFT)
MCF7 (Dox+ 1μM VOR)
MCF7 (Dox+ 2μM VOR)
CI
Fraction Affected

E

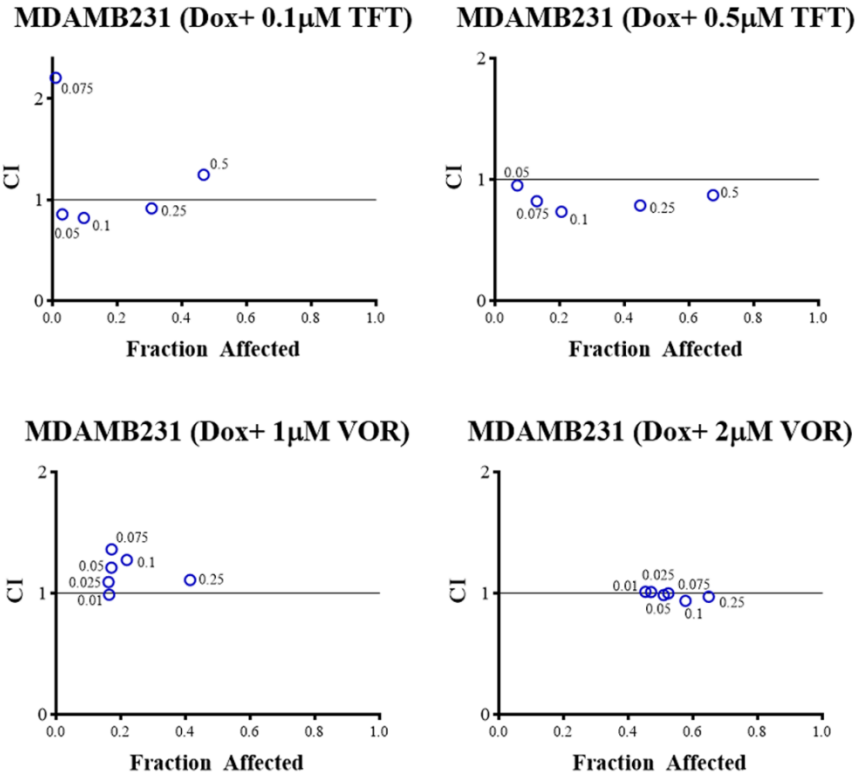
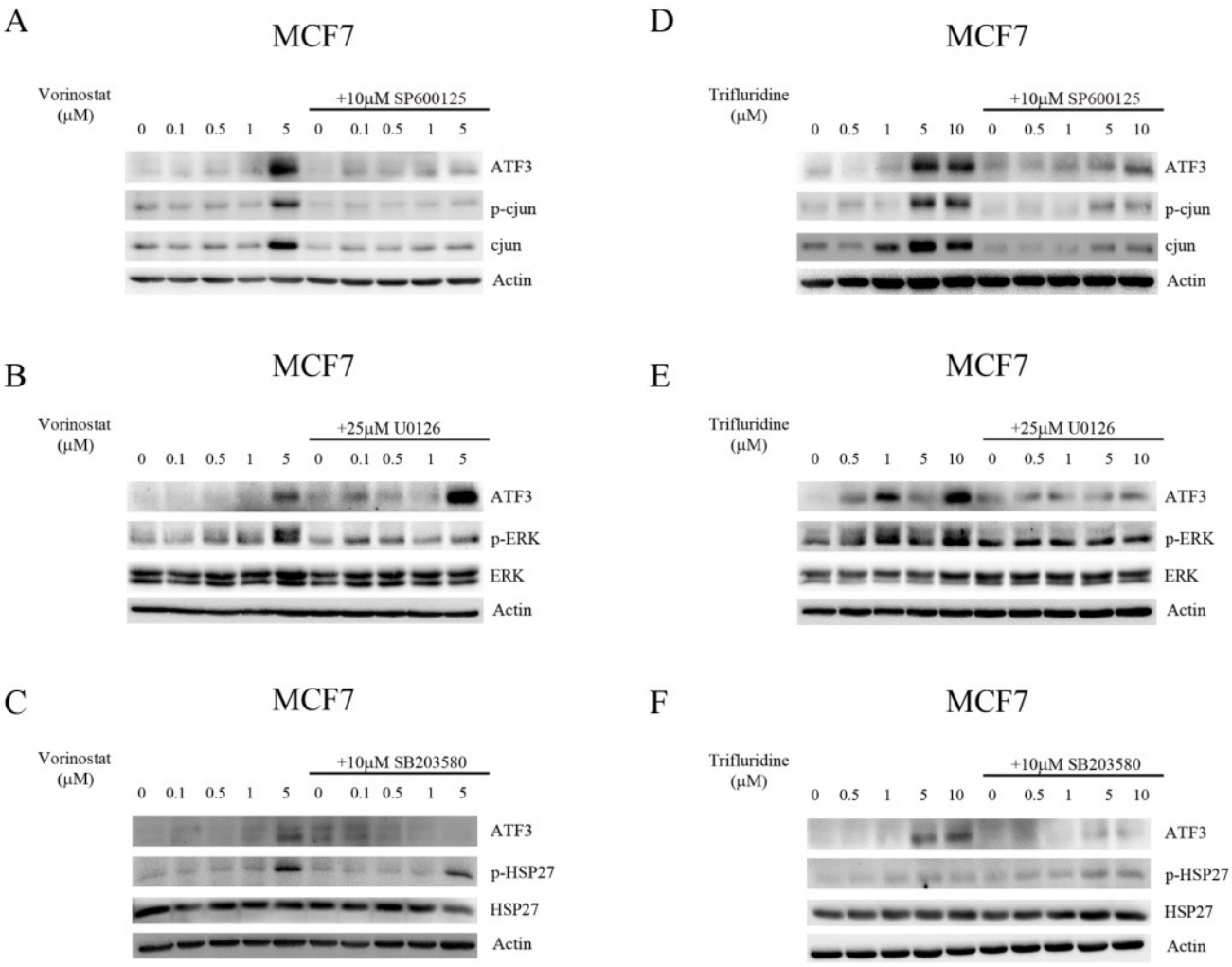

MDAMB231 (Dox+ 0.1μM TFT)
MDAMB231 (Dox+ 0.5μM TFT)
MDAMB231 (Dox+ 1μM VOR)
MDAMB231 (Dox+ 2μM VOR)
CI
Fraction Affected

Figure 17: A-C) Western blot analysis for ATF3 expression following 24 hour vorinostat treatment with chemical inhibitors for A) JNK (SP600125), B) ERK1/2 (U0126), C) p38 (SB203580) in the MCF7 cells. D-F) Western blot analysis for ATF3 expression following 24 hour vorinostat treatment with chemical inhibitors for D) JNK (SP600125), E) ERK1/2 (U0126), F) p38 (SB203580) in the MCF7 cells


A
MCF7
Vorinostat (μM)
+10μM SP600125
0 0.1 0.5 1 5 0 0.1 0.5 1 5
ATF3
p-cjun
cjun
Actin
D
MCF7
Trifluridine (μM)
+10μM SP600125
0 0.5 1 5 10 0 0.5 1 5 10
ATF3
p-cjun
cjun
Actin
B
MCF7
Vorinostat (μM)
+25μM U0126
0 0.1 0.5 1 5 0 0.1 0.5 1 5
ATF3
p-ERK
ERK
Actin
E
MCF7
Trifluridine (μM)
+25μM U0126
0 0.5 1 5 10 0 0.5 1 5 10
ATF3
p-ERK
ERK
Actin
C
MCF7
Vorinostat (μM)
+10μM SB203580
0 0.1 0.5 1 5 0 0.1 0.5 1 5
ATF3
p-HSP27
HSP27
Actin
F
MCF7
Trifluridine (μM)
+10μM SB203580
0 0.5 1 5 10 0 0.5 1 5 10
ATF3
p-HSP27
HSP27
Actin

Chapter 5: Discussion

In the present study, we further characterized and established the role of the stress-inducible gene ATF3 as a regulator of DNA-damaging chemotherapy cytotoxicity and also established ATF3 as a therapeutic target for the treatment of NSCLC and breast cancer. Our laboratory had previously demonstrated that ATF3 plays an important role in regulating cisplatin cytotoxicity [209] including showing that shRNA knockdown of ATF3 in A549 lung cancer cells reduces sensitivity to cisplatin treatment (Appendix Figure 20) [209].

Lung cancers are by far the leading cause of cancer morbidities and mortalities in Canada [1]. NSCLC is the most prevalent form of lung cancer accounting for approximately 85% of tumors [30]. Platinum based chemotherapy is currently the standard of care for patients with non-resectable NSCLC, and usually consists of a combination of cisplatin with other chemotherapies including paclitaxel, docetaxel, and gemcitabine [274]. These combinations have been identified empirically and the underlying mechanisms are not well understood. The use of cisplatin is also limited due to various toxicities associated with treatment, such as nephrotoxicity, as well as acquired resistance to treatment. Intrinsic or acquired resistance to cisplatin results in patients being exposed to the toxicities of cisplatin treatment without obtaining clinical benefit. In addition, a small proportion of patients remain progression free after 5 years demonstrating the need for alternative therapeutic strategies. Clearly novel combination based strategies are urgently needed.

5.1 ATF3 as a Regulator of Chemotherapy Response in NSCLC and Breast Cancer

In this study, we established cisplatin-resistant cells using the commonly used NSCLC derived Calu6 and H23 cell line models. RNA-seq analysis on these cell lines identified ATF3 as a highly significant, differentially induced gene in the parental cell lines (Calu6 and H23) compared to the cisplatin-resistant (CisR1) sublines following cisplatin treatment. Cisplatin strongly induced ATF3 expression, both at the mRNA and protein level, in the parental cell lines but induction of ATF3 expression was abrogated in the cisR1 sublines. Loss of ATF3 expression was demonstrated to reduce the sensitivity of ATF3 deficient MEFs to cisplatin as well as other DNA-damaging chemotherapeutics carboplatin, doxorubicin, and trifluridine, indicating that ATF3 plays a role in mediating the cytotoxic response to a variety of DNA-damaging agents that are widely employed anti-cancer agents [275].

ATF3 was identified in this thesis as the most differentially expressed apoptotic effector upon cisplatin treatment of the Clau6 and H23 NSCLC cell lines compared to their paired cisplatin-resistant sub-clones through RNA-seq analysis. Seventy-five genes were identified to be commonly dysregulated between the two sets of parental cell lines and a representative cisplatin resistant sub-line following cisplatin treatment (Appendix Table 1). Gene Ontology (GO) analysis identified a significant enrichment of genes with GO Terms “regulation of signal transduction” and “signal transduction” (Appendix Table 3) and the top five dysregulated genes are shown in Appendix Table 2. Of interest, is that *FOSB* was identified among the top five differentially regulated genes in the cisplatin-resistant cells compared to their respective parental lines. *FOSB* encodes a leucine-zipper protein that forms dimers with JUN to comprise the AP-1

transcription factor [188]. FOSB can also dimerize with ATF3 [206]. A role for FOSB in regulating cell death in breast cancer has previously been demonstrated where upregulation of FOSB expression in TNBC cells leads to cell death [276]. Conversely, it has also been demonstrated that the expression of Δ*FOSB*, a splice variant of *FOSB*, protects MCF7 cells from cell death [277]. Since ATF3 and FOSB have both been demonstrated to be involved in cell death and/or survival and can form heterodimers with each other it would be of interest to determine the role of ATF3/FOSB dimers in mediating the cytotoxic response to cisplatin. Based on the RNA-seq data, the inability to induce *FOSB* and *ATF3* expression in the cisplatin resistant sub-clones could suggest that they interact to mediate cell death or survival. Further investigation in to the role of FOSB and the ATF3/FOSB heterodimer may demonstrate the importance of these two proteins for mediating the cisplatin cytotoxicity in the NSCLC cells.

Further characterizations of our cisplatin-resistant NSCLC cell lines revealed that JNK signaling specifically was impaired and that the exogenous expression of an activated JNK construct restored ATF3 expression and re-sensitized the cisR1 sub-lines to cisplatin cytotoxicity. This data further demonstrated that the dysregulation of ATF3 was a key driver of cisplatin resistance. Taken together, our data demonstrates that ATF3 is a key mediator of cisplatin response and that loss or dysregulation of ATF3 results in resistance to cisplatin treatment in NSCLC cells.

Since ATF3 expression can be induced by multiple stress stimuli and various singling pathways, we evaluated the potential of other chemotherapeutics to induce ATF3 expression in these cisplatin resistant sub-lines. Treatment with docetaxel, a microtubule-targeting agent) failed to significantly induce ATF3 expression, while doxorubicin treatment strongly induced ATF3

expression in the cisR1 cells. The cisR1 cells also demonstrated comparable sensitivity to the parental cells when treated with doxorubicin. This demonstrates that the mechanism of resistance of the cisplatin-resistant cells is due to the dysregulation of signaling pathways involved in regulating ATF3 expression but that these cells are not deficient in either their ability to induce apoptosis or induce ATF3 expression through alternative mechanisms.

These observations led to our studies evaluating the role of the induction of ATF3 expression as a regulator of doxorubicin cytotoxicity in breast cancer cells as this agent is a key treatment option in this tumor type [47]. Breast cancer cell lines, MCF7 and MDAMB231, and prostate cancer cell lines, PC3 and LN-CaP as comparators (Appendix Figure 19), all demonstrated increased ATF3 expression following treatment with cisplatin and doxorubicin. Additionally, we demonstrated for the first time that these DNA-damaging chemotherapeutics can induce ATF3 expression in surgically excised human tumor specimens. Taken together these data demonstrate that ATF3 expression is induced by DNA-damaging agents in a broad and clinically relevant context and represents a novel therapeutic target for the treatment of cancer. It also provides the initial rationale for the potential use of ATF3 as a predictive biomarker of chemotherapy response.

The use of *ex-vivo* tissue provided a more clinically relevant model for the assessment of ATF3 induction following treatment with cisplatin or doxorubicin. This model provided a system in which several tumor samples could be assessed for their ability to upregulate ATF3 expression following chemotherapy treatment. Although evaluated *ex-vivo*, the architecture of the tumor discs with minimal manipulation is a more relevant tumor model that tumor derived cell lines. As observed in our studies, not all tumor samples upregulated ATF3 expression following

cisplatin or doxorubicin treatment. This suggests a differential inter-patient response to these drugs in lung and breast or ovarian cancers, respectively as observed in the clinic. These findings are preliminary and sample size is low, but the differential upregulation of ATF3 upon cisplatin or doxorubicin treatment observed could represent a novel predictor of response. Future studies could evaluate the ability of cisplatin or doxorubicin to induce ATF3 expression in tumors *ex-vivo* prior to patients receiving treatment and then follow patient response with respect to tumor size and overall survival. This would determine whether the differential induction of ATF3 in patient tumor samples represents a predictor of chemotherapy response. While these drugs could induce ATF3 expression in some of the tumors, there are potential caveats to this system. Firstly, the tissue samples obtained were small, which limited the investigation of multiple concentrations and time points following treatment. The tumor tissues were processed by the pathology department at the Ottawa Hospital and we obtained tumor tissue resected from the centre of the tumor mass, but we did not examine the tumor content of the tissue sample obtained. Therefore, the ability of the drugs to induce ATF3 expression in the tumor cores could be affected by cellular content of the tissue tested. On the other hand, the heterogeneity of the processed tumor cores may better represent the natural state of the tumor, as it is well accepted that these tumors are heterogeneous [278]. Examining the tumor content of the tissue samples obtained for future studies will be beneficial for better interpretation of the data obtained.

ATF3's role in promoting cell death and/or survival in cancer has been unclear, with studies suggesting a context dependent role for ATF3. Depending on cancer type, stress stimuli, or cellular mechanisms involved, ATF3 has been demonstrated to promote or supress tumor growth. In our study we clearly demonstrate that ATF3 plays an important role in mediating the

cytotoxicity of DNA-damaging agents including in MEFs, since loss of ATF3 significantly reduced the sensitivity of the MEFs to both of these drugs. This reduction was not caused by an overall impairment in cellular apoptosis since the ATF3 knockout cells displayed similar sensitivity to docetaxel whose mechanism of cytotoxicity is independent of ATF3 expression. In our cisplatin-resistant cell lines, their inability to induce ATF3 expression following cisplatin treatment resulted in reduced sensitivity. These findings suggest that targeting ATF3 to enhance and sustain its expression may represent a viable combination-based therapeutic strategy for the treatment of NSCLC and breast cancer.

In our study, doxorubicin similarly induces ATF3 expression in cisplatin-resistant NSCLC cells and importantly in breast cancer cells and surgically excised tumors *ex-vivo*. Similar to cisplatin, doxorubicin is also a potent DNA-damaging agent and it was of interest to determine the role of ATF3 in mediating its cytotoxic effect. Few studies have looked at the role of ATF3 in mediating doxorubicin cytotoxicity. Previously, it was demonstrated that loss of ATF3 prevents cell death of the kidney proximal tube cell line, HK2, when treated with doxorubicin [211]. But in another study, overexpression of ATF3 protected cardiomyocytes from doxorubicin induced cytotoxicity [279]. These contradicting finding further demonstrate the context dependency of ATF3 function. Since ATF3, can both act as a regulator of response and recovery to cellular stressors and can trigger apoptosis if the stress cannot be overcome: variations in the cellular context, the nature and duration of the stress will play a role in the overall cellular response.

The data presented in this thesis suggests that there is a positive correlation between increased ATF3 expression and cell death upon treatment with cisplatin or doxorubicin through driving its

apoptotic response. ATF3 expression was found to be strongly upregulated by both drugs prior to observing significant cell death. A time dependent increase in ATF3 expression was observed up to 24 hours at doses of drug that display significant cytotoxicity at 48 hours. But, the role of ATF3 as a definitive mediator of the apoptotic response to cisplatin and doxorubicin requires further investigation. While loss of ATF3 in MEFs resulted in a drastic reduction in sensitivity to both cisplatin and doxorubicin, this model does not best represent cancer, as the MEFs are untransformed. The attempt to knockout ATF3 in the breast cancer cell line MCF7 failed to completely recapitulate what was observed in the MEFs, with a relatively weaker difference in sensitivity observed only at low concentration of doxorubicin. This work was performed in a single sub-clone from the CRISPR knockout and therefore could be the result of clonal heterogeneity during the selection process. Conversely, MCF7 cells possess a wide range of genetic abnormalities altering a variety of cellular signalling pathways that may result in a needed but reduced requirement for ATF3 in regulating apoptosis. This requires further investigation. In the NSCLC models employed in this thesis, ATF3 induction was attenuated in the cisR1 cells following cisplatin treatment. The inability of cisplatin to induce ATF3 expression was also correlated with reduced sensitivity to cisplatin treatment. While restoring ATF3 expression through the use of an activated JNK construct re-sensitized the resistant cells to cisplatin, this did not confirm that the cytotoxic effect was mediated through ATF3. Since JNK signaling was dysregulated in the cisR1 cells, by reintroducing an activated JNK plasmid multiple signaling pathways would be affected that could be important in mediating cisplatin cytotoxicity. To definitively assess the importance of ATF3 in mediating the cytotoxicity of cisplatin in the NSCLC cells, it would have been beneficial to directly re-express ATF3 in the

cisR1 cells and determine whether this results in the restoration of sensitivity to cisplatin treatment.

Interestingly, it has been suggested that ATF3 plays an important role in mediating apoptosis in early stage cancers [229, 235]. *Yin X et al* demonstrated that ATF3 played an important role in mediating cell death in normal-like MCF10a cells, where as it was protective in the more aggressive MCF10CA1a cells [235]. *Wang Z et al* also demonstrated that loss of ATF3 may contribute to the early formation of tumors [229]. Therefore, it is possible that the role of ATF3 as a mediator of either recovery from stressors or apoptosis is dependent on the malignancy of the cell, and this may explain why significant reductions in sensitivity to cisplatin and doxorubicin were observed in the MEFs compared to when ATF3 was knocked-out in the MCF7 cells. Conversely, it may be possible that ATF3 is playing an anti-apoptotic role following treatment with cisplatin and doxorubicin. A recent study by *Zhoa W et al* reported that ATF3 played an important role in mediating radioresistance in breast cancer cells through the regulation of the PI3K/Akt pathway [280]. Further studies evaluating the potential anti-apoptotic role of ATF3 in breast cancer are required to better understand the complex nature of ATF3's function.

5.2 ATF3 and the DNA-Damage Response

Multiple signaling mechanisms are activated following DNA-damage to initiate the DDR. The Phosphatidylinositol-3 kinase- like protein kinases (PIKKs) are considered to be the initial kinases activated upon DNA-damage [281]. This family of proteins includes ATM, ATR, and

DNA-PK, which respond to different forms of DNA-damage [273]. Previous reports have demonstrated that ATM and ATR are activated upon treatment with cisplatin [282] and doxorubicin [283]. We demonstrated in breast cancer cell lines that inhibition of ATM results in an attenuation of ATF3 induction following treatment with doxorubicin. Since doxorubicin is a potent inducer of double-strand DNA breaks, ATM may be an important kinase responsible for ATF3 induction following doxorubicin treatment.

Previous reports have demonstrated a role for ATF3 in mediating the cellular response to DNA-damage as loss of ATF3 results in an increase in genomic instability [229]. Wang Z et al demonstrate that loss of ATF3, in MEFs, caused a decreased DNA repair capacity following ionizing radiation treatment [229]. Similarly, downregulation of ATF3 resulted in the accumulation of DNA-damage in human cells [284]. Furthermore, impaired ATM activation was observed in ATF3 knockout MEFs [229] suggesting that ATF3's role in mediating DNA-repair is partly regulated through regulating ATM activation. Following the formation of double-strand DNA breaks, ATM is acetylated at K3016, by Tip60, which allows for ATM's autophosphorylation and activation [285]. ATF3 has been demonstrated to bind to Tip60 and increase its activity thus promoting ATM signaling upon DNA-damage [286]. Since ATM is involved in upregulating ATF3 expression and ATF3 is involved in regulating ATM activity, this suggests a positive feedback loop that may be initiated during the DDR.

The function and role of ATF3 during the DDR has also been strongly linked to p53. ATF3 has been demonstrated to directly interact with p53 and by doing so is able to stabilize p53 expression by preventing MDM2 mediating ubiquitination and subsequent proteasome-mediated

degradation [213]. Of the cancer cell lines employed throughout this thesis, the MCF7 cells were the only ones to express wild-type p53. While we do not know the p53 status of the immortalized MEFs, it has been demonstrated that mutations in p53 are very common during the immortalization process [287]. A recent study by *Wang Z et al* demonstrated the importance of ATF3 in maintaining genome integrity [229]. They demonstrated that ATF3 knockout MEFs are genetically unstable and display aberrant chromosomes and micronuclei. This genetic instability was dependent on the regulation of p53 by ATF3 [229]. ATF3 knockout mice display increased spontaneous tumorigenesis and decreased survival [229]. Additionally, loss of ATF3 promotes tumor formation in $p53^{+/-}$ but not in $p53^{-/-}$ mice [229]. The work by *Wang Z et al* has demonstrated that ATF3 is an important factor responsible for maintaining proper p53 function and this may be through ATF3 transcriptional regulation of p53 and/or stabilization of p53 protein expression.

Sustained and elevated expression of ATF3 leads to cellular apoptosis [208]. Throughout this thesis, the induction of ATF3 expression by various chemotherapies was shown to be time and dose dependant. Whether elevated ATF3 expression is sustained over time or fluctuates throughout the time points investigated warrants further investigation. Since ATF3 can repress its own expression, it is plausible that ATF3 expression is upregulated and then acts to negatively feedbacks on its own expression, but this oscillation could repeat due to persistence of the cellular stress. This would be similar to what has been observed with the expression of p53 following ionizing radiation (IR) [288]. p53 expression was demonstrated to oscillate following treatment with IR. The number of oscillations was shown to be related to p53 cellular function during the stress response, with p53 regulating the expression of genes involved in cellular arrest

during the first four oscillations and apoptosis with the subsequent oscillations [288]. Because ATF3 has been demonstrated to interact and cooperate with p53, future work should investigate whether ATF3 follows a similar oscillatory expression profile and its potential role in regulating p53's oscillatory expression. It would also be beneficial to determine whether ATF3 function is dependent of the p53 status of tumor cells. This is of significant interest as it may suggest a new paradigm of drug synergy where targets that can regulate cell responses to stressors through cell recovery pathways can be driven to apoptosis through enhanced and sustained expression by combining inducers through different stimuli.

5.3 ROS and ATF3 Induction by Cisplatin and Doxorubicin

It has been well documented that treatment with platins and anthracyclines generate ROS. ROS generated through cisplatin treatment mainly occurs through mitochondrial dysfunction [289]. While doxorubicin generated ROS occurs through the redox cycling of its quinone moiety [101, 104]. ROS are attributed as one of the mediators of the cytotoxic effect of both cisplatin and doxorubicin as it can contribute to metabolic dysfunction and increased DNA-damage. In this thesis, the upregulation of ATF3 expression following doxorubicin treatment was attenuated when MCF7 cells were pretreated with NAC, which demonstrates the role of ROS in mediating doxorubicin induced ATF3 expression. NAC treatment was also previously demonstrated to reduce cisplatin and doxorubicin cytotoxic and apoptotic effect [290, 291]. NAC is a precursor to glutathione which acts as an antioxidant within cells. While NAC treatment reduces ROS species generated by cisplatin and doxorubicin treatment it may also play a role in mediating the detoxification of these drugs, thus preventing their cytotoxic effect. Elevated expression of

glutathione is linked to cisplatin resistance in *in vitro* and *in vivo* models [80, 82, 99]. Doxorubicin cytotoxicity was also demonstrated to be inhibited by the addition of extracellular glutathione in leukemic cells [292]. While cisplatin can be conjugated to glutathione for its detoxification, it was demonstrated that doxorubicin-glutathione conjugates do not form in MCF7 cells [293]. This suggests that the reduction in ATF3 expression observed when MCF7 cells were pretreated with NAC is a result of reduced ROS rather than an interaction between doxorubicin and NAC that could alter doxorubicin activity.

Throughout this thesis MTT assay was employed as a measure of cellular viability following treatment with cisplatin, doxorubicin, or the various combinations. The MTT assay is a colorimetric assay that is widely employed as a measure of cell viability, but has the main limitation of being dependent on the metabolic activity of the cells. Therefore, compounds that effect cellular metabolism, specifically mitochondrial activity, such as ROS can result in variations in the measure of cellular viability. To help confirm the MTT results, Western blot for the cleavage of PARP was employed as another measure of cellular apoptosis. The cell viability data obtained by MTT assay could also be furtherconfirmed through the use of trypan blue exclusion assays, live cell imaging or flow cytometry.

5.4 JNK Signaling Pathway and ATF3 Expression

The role of the MAPK signaling pathways have also been widely studied in response to DNA-damage [294, 295]. In this thesis, it was determined that JNK plays an important role in

mediating cisplatin and doxorubicin's ability to induce ATF3 expression. Inhibition of JNK resulted in attenuated ATF3 expression in the breast cancer cells following doxorubicin or cisplatin treatment. Previously our laboratory demonstrated that inhibition of JNK attenuated cisplatin induced ATF3 expression and reduced the sensitivity of A549 NSCLC cells to cisplatin treatment [209]. Presented in this thesis, JNK signaling was demonstrated to be disrupted in the cisplatin-resistant NSCLC cell lines which contributed to their mechanism of resistance. Re-expression of an activated JNK chimeric induced ATF3 expression and re-sensitized the resistant cell lines to cisplatin treatment. These findings demonstrate a clear role for JNK in upregulating ATF3 expression following treatment with cisplatin or doxorubicin. Interestingly, while JNK was involved in mediating ATF3 expression following cisplatin or doxorubicin treatment, the inhibition of the other MAPK singling pathways only affected cisplatin induced ATF3 expression in the cell lines tested. This suggests that ATF3 expression is regulated by various MAPK signaling pathways dependent on cellular context and the type of DDR initiated.

JNK signaling has been implicated in both pro-apoptotic and cell survival roles following DNA-damage. *Kim MJ et al* demonstrated that inhibition of JNK reduces the sensitivity of cervical cancer cells to ionizing radiation [296]. They also demonstrated that in cancer cells treated with ionizing radiation JNK could regulate Bax and Bak activation as well as the down-regulation of Bcl-2 and contribute to mitochondrial apoptotic cell death [297]. Additionally, it has been suggested that JNK activation following DNA-damage by ionizing radiation may be regulated by ATM [298]. The pro-survival role of JNK to DNA-damage has been demonstrated to be regulated through its downstream target c-Jun and related transcription factor ATF2. Inhibition

of JNK sensitized breast cancer cells to various genotoxic agents, including cisplatin and etoposide, and was attributed to decreased activation of ATF2 [299]. Similarly, JNK mediated phosphorylation of c-Jun was demonstrated to help protect cancer cells from a panel of DNA-damaging agents, including cisplatin and doxorubicin [300].

JNK signaling leads to the activation of AP-1 and upregulation of ATF3 expression [210]. Subsequently, ATF3 forms heterodimers with other ATF/CREB family members to activate or repress transcription. A number of potential dimers can be formed with ATF3 and this is highly dependent on the expression of its ATF/CREB protein binding partners. The transcriptional regulation, whether it is activation or repression, is also dependent on the binding partner that forms the heterodimer with ATF3 or homodimers. In addition the heterodimers have a different repertoire of gene regulation that can lead to apoptosis [200, 201]. This suggests that ATF3 function during cellular stress response is highly context dependent. It is possible that the dual role of ATF3 following stress is regulated by which binding partners are available, and that as cellular stress persists the profile of available binding partners changes to those that are involved in regulating apoptosis rather than recovery. It has been demonstrated in breast cancer that the expression of various AP-1 family members, Jun and Fos proteins, is altered between normal tissue and adjacent tumor, as well as between histopathological subtypes [301]. The difference in the expression pattern of these ATF3 binding partners may also determine the role that ATF3 plays in mediating the apoptotic response to cellular stress.

5.5 ATF3 Inducing Compounds Enhance the Cytotoxicity of DNA-Damaging Chemotherapeutics

Sustained and enhanced expression of ATF3 can initiate an apoptotic response following cellular stress. Since ATF3 is readily upregulated following cellular stress and involved in mediating the cytotoxic effect of cisplatin and doxorubicin, it may represent a target for combination therapeutic strategies to enhance current treatments. A 1200 FDA-approved compound library screen comparing responses in $ATF3^{+/+}$ to $ATF3^{-/-}$ MEFs was performed in this study to identify clinically relevant agents whose mechanisms of cytotoxicity were dependent on the presence of ATF3. A second screen to identify agents that specifically enhanced the cytotoxicity of doxorubicin in MCF7 cells was also performed. Vorinostat, trifluridine, and 6-mercaptopurine were identified as hits in both screens and were further evaluated.

Trifluridine, a fluoropyrimidine, has demonstrated potent anticancer activity but due to poor bioavailability its use has been limited. Trifluridine is rapidly phosphorylated to produce its active monophosphate form which is incorporated into DNA resulting in cell cycle arrest which is attributed to be the primary mechanism of trifluridine cytotoxicity [302, 303]. Recent advances have demonstrated that the combination of trifluridine with a thymidine phosphorylase inhibitor (tipiracil) can greatly enhance its bioavailability and efficacy [155, 304, 305]. Trifluridine /tipiracil (TAS-102) treatment has recently been FDA- approved for the treatment of metastatic colorectal cancer. The combination of trifluridine and doxorubicin demonstrated synergistic cytotoxicity in the luminal breast cancer cell line, MCF7, but not in the triple-

negative line, MDAMB231. Previously, MDAMB231 cells have been demonstrated to be less sensitive to 5-flurouricil, which has a similar mechanism of action to trifluridine [306]. Additionally, MDAMB231 cells express elevated RNA levels for thymidylate synthase [307] which may confer some resistance to trifluridine if the protein expression is also elevated [308]. Further evaluation of the combination of doxorubicin and trifluridine is required, and in future studies it would be beneficial to evaluate the combination using TAS-102.

Vorinostat is a HDAC inhibitor that has been approved for the treatment of cutaneous T-cell lymphoma, and is being assessed in the treatment of other cancers. HDAC inhibitors have demonstrated preclinical and clinical activity as single agents and in combination with other chemotherapies, such as platins [309-311]. A Phase 1 clinical trial assessed the combination of vorinostat and doxorubicin and demonstrated that the combination displays some clinical benefit for the treatment of solid tumors [312]. Due to lack of specificity and off-target toxicities associated with treatment, the clinical use of HDAC inhibitors is limited [313]. In our study the combination of vorinostat with doxorubicin had an additive effect on the MCF7 breast cancer cells. Similar to what was observed with trifluridine, combination of vorinostat and doxorubicin did not enhance tumor cell killing in the MDAMB231. This finding was also recently demonstrated by *Tarasenko et al* who demonstrated that the combination of multiple HDACi, including vorinostat, with doxorubicin resulted in an antagonistic combination [314]. Of interest, this study found that the combination of the HDACi valporic acid and AN446 did synergistically enhance doxorubicin cytotoxicity in MDAMB231 cells [314]. This suggests that HDAC inhibition can still be used as a potential combination therapeutic strategy with doxorubicin, but is dependent on the specific HDACi employed. The investigation of the

differences in the mechanisms of action of VPA and AN446 compared to vorinostat may provide novel insight in to the differential activity of HDAC inhibitors with doxorubicin.

A synergistic enhancement in cytotoxicity was observed in the combination of vorinostat with cisplatin in the NSCLC tumor cells. Of interest, was that this combination displayed synergistic cytotoxicity in the cisplatin-resistant NSCLC cell lines suggesting the potential for this combination in treating cisplatin-resistant tumors. Previous studies have demonstrated that HDACi can enhance the cytotoxicity of cisplatin [315-320] and doxorubicin [321-325] in a number of cancer models. While the enhancement in cytotoxicity of these combinations has been well established, the downstream mechanisms are not well defined. HDACi has been demonstrated to initiate the ISR which may be the mechanism responsible for the enhanced cytotoxicity observed [326, 327]. Other reports have demonstrated the role of p53 and the mitochondrial apoptotic pathway [319], as well as the potentiation of DNA-damage and ROS production [318] as the mechanism for the observed enhancement in cytotoxicity.

Vorinostat treatment also prevents the deacetylation of histones which results in open and more accessible chromatin for DNA-damaging drugs to target. All of these pathways are able to induce ATF3 expression and may be responsible for how vorinostat treatment upregulates ATF3 expression. In the cisplatin-resistant cells, the ability of vorinostat to induce ATF3 expression through an alternate pathway other than JNK may explain how vorinostat treatment is able to re-sensitize the cisplatin-resistant cells to cisplatin treatment. This provides rationale for the targeting of ATF3 as a potential therapeutic target. Through combination approaches with drugs that can upregulate ATF3 through various mechanisms, elevated and sustained expression levels

of ATF3 can be obtained to potentially increase tumor cell cytotoxicity. Identifying novel and more specific ATF3 inducers may be useful in the development of future combination therapies.

Future studies investigating the effect of enhanced and sustained ATF3 expression will provide further insight in to the potential of this therapeutic approach. For example, a controlled expression system, such as a doxycycline inducible ATF3 expression system, should be used to determine how varying cellular ATF3 expression levels affects tumor cell sensitivity to doxorubicin or cisplatin.

Another possible explanation for the synergistic effect of the combination of vorinostat or trifluridine with doxorubicin is that these compounds may affect chromosomal stability which was previously demonstrated to predict response to doxorubicin treatment [117, 118]. In our cell culture models, pre-treatment with trifluridine or vorinostat prior to doxorubicin treatment may have affected chromosomal integrity through various mechanisms. There is evidence which suggests that HDAC inhibitors and nucleoside analogues can promote chromosomal instability. It was demonstrated that inhibition of HDAC, with trichostatin A, resulted in increased chromosomal instability through the prolongation of mitotic arrest and improper mitotic checkpoint protein localization [328]. Trifluridine treatment induces a mismatch repair DNA-damage response which may sensitize tumor cells to subsequent doxorubicin treatment. Additionally, the nucleoside analogue 5-fluro-2-deoxyuridine was demonstrated to incorporate into telomeres which promoted genomic instability and cell death [329]. Since it was demonstrated that chromosomal instability can predict response to doxorubicin treatment, the combination of drugs that can increase chromosomal instability with doxorubicin may provide a

beneficial therapeutic approach and help sensitize tumors to doxorubicin treatment. Here we identified clinically relevant agents that could be used in combination therapies with doxorubicin that can potentially induce chromosomal instability to enhance doxorubicin efficacy.

Mutations in DNA-repair pathways could also explain the synergistic effect of the combination treatments identified in this thesis. For example, *BRCA1* and *BRCA2* mutations have been identified to be associated with response to DNA-damaging chemotherapy. These genes encode for proteins that play an important role in mediating DNA-damage repair through homologous recombination. In breast cancer, mutations in *BRCA1* and *BRCA2* are associated with sensitivity to DNA-damaging chemotherapies, such as platins and PARP inhibitors [67]. In a recent phase II clinical trial it was demonstrated that single agent platin treatment displayed significant activity in *BRCA1* and *BRCA2* germline mutation carriers [330]. In the present study the MCF7 and MDA-MB-231 breast cancer cell lines employed express wild-type BRCA1 and BRCA2 [331, 332]. Therefore, the effects of the combination treatments identified are not a result of an impaired DNA-damage response as a result of mutated BRCA1/2 in these cell lines. While trifluridine primarily induces a mismatched repair response, it has been demonstrated that vorinostat treatment can lead to double-stranded DNA breaks [180]. The use of HDAC inhibitors has also been demonstrated to augment DNA damage response and reduce expression of homologous recombination proteins which can sensitize breast cancer cells to cisplatin or PARP inhibitors [333]. This may explain why vorinostat enhances cisplatin and doxorubicin treatment in wild-type BRCA1/2 cells. Since trifluridine and vorinostat can induce DNA-damage, future studies investigating the activity of these combinations in the context of other impaired DDR pathways would help to provide a better understanding of the utility of these

combination therapies, as well as potentially identify patients who would be responsive to these treatment options.

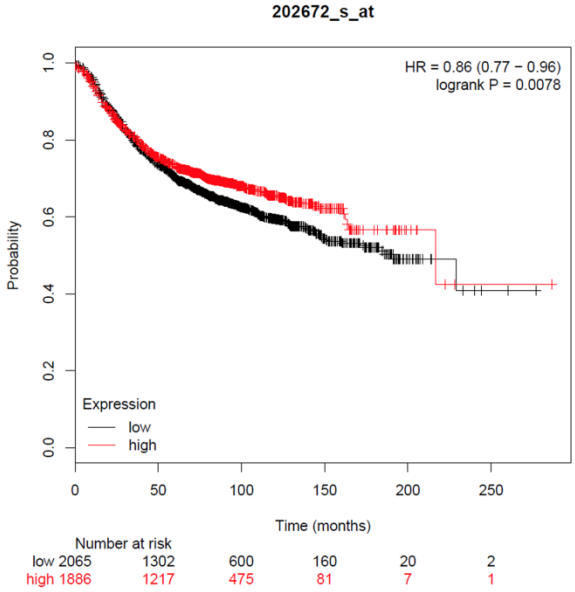
5.6 Conclusion

This thesis characterized the role of ATF3 in mediating the cytotoxic effect of DNA-damaging chemotherapies, cisplatin and doxorubicin. While these drugs induce DNA-damage through different mechanisms, both were potent inducers of ATF3 expression. Interestingly, ATF3 was shown to play an important role in mediating the cytotoxicity of these drugs in immortalized MEFs. In the NSCLC cells, inability to induce ATF3 expression was correlated with reduced sensitivity to cisplatin, and in the MCF7 breast cancer cells knockout of ATF3 correlated with reduced but not as robust reduced sensitivity to doxorubicin treatment. This data suggests a context dependent role of ATF3 in mediating cell death which requires further investigation. Interestingly, there is evidence to suggest that breast and lung cancer patients with elevated expression of ATF3 have better relapse-free survival than patients that have lower expression of ATF3, suggesting there may be potential for the use of ATF3 as a predictor of chemotherapy response (Figure 18). Furthermore, we identified and demonstrated that the combination of ATF3 inducing agents with cisplatin or doxorubicin can enhance their cytotoxic effect (Figure 19). While it remains unclear as to whether ATF3 is mediating the cytotoxicity of the combination treatments, the screening strategy employing ATF3 was successful in identifying agents that can be used to enhance the efficacy of cisplatin and doxorubicin. Together the data presented in this thesis provide a rationale for targeting ATF3 in potential combination therapeutic strategies.

Figure 18: Kaplan-Meier survival curves for high and low ATF3 expression in breast and lung cancer patients. A) Survival data for all breast cancer patients. B) Survival data for breast cancer patients who received adjuvant chemotherapy. C) Survival data for all lung cancer patients. Data was obtained from the Kaplan-Meier plotter. Breast [334] Lung [335]

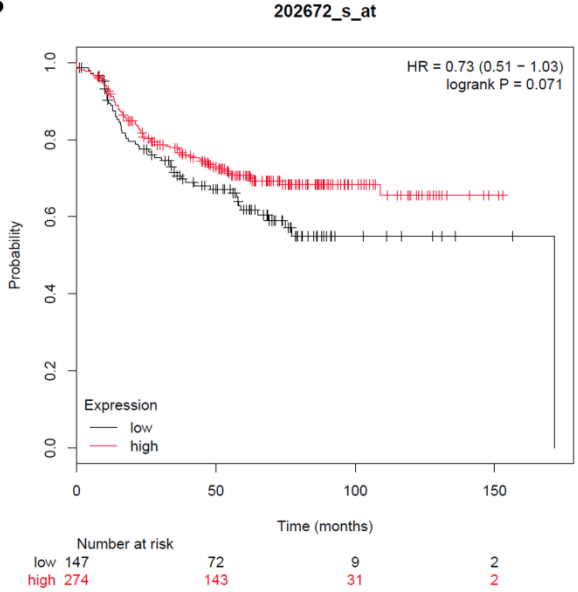
A

All Breast Cancer Patient



B

Adjuvant Chemotherapy



C

All Lung Cancer Patients

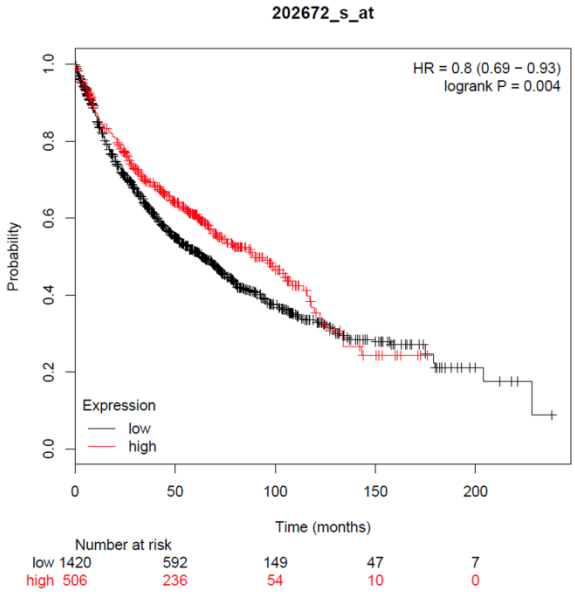
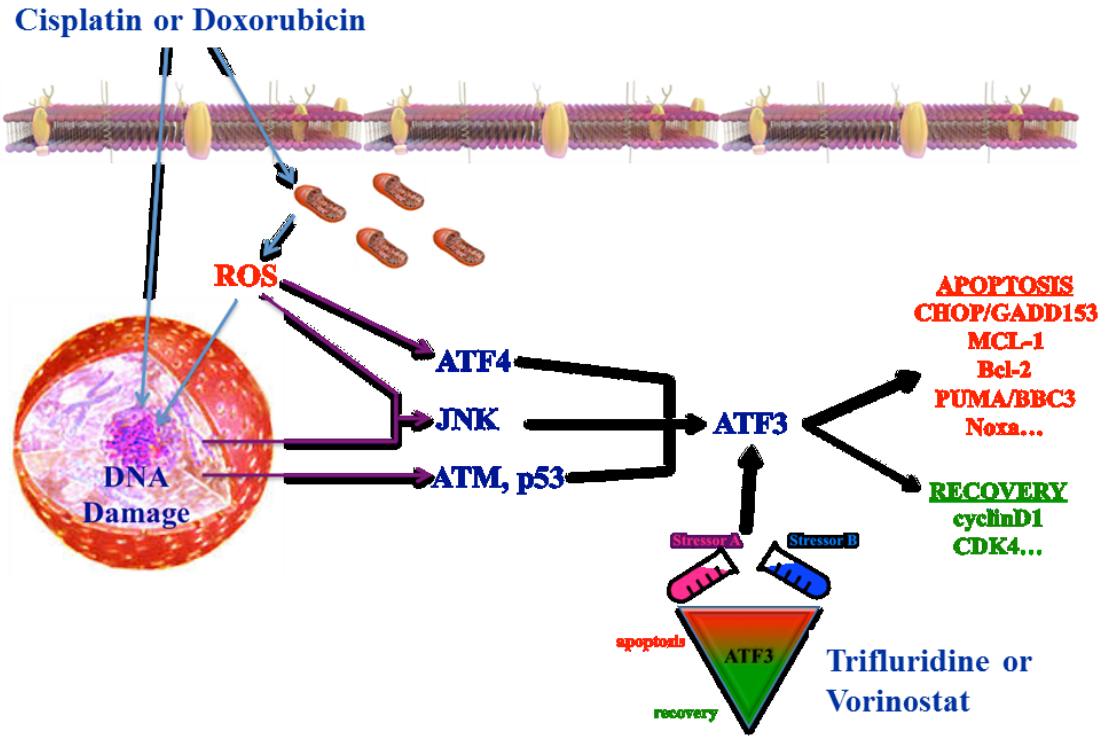


Figure 19: Schematic demonstrating how cisplatin and doxorubicin upregulate ATF3 expression in breast or lung cancer cells. Treatment with these chemotherapies upregulates ATF3 expression through a number of pathways mediated by DNA-damage and ROS. We demonstrated that the use of other ATF3 inducing compounds, such as trifluridine or vorinostat, can synergize with cisplatin or doxorubicin. We propose that this is a result of the ability of the combination treatment to elevate and sustain ATF3 expression in order to promote its pro-apoptotic function.


Cisplatin or Doxorubicin
ROS
DNA
Damage
ATF4
JNK
ATM, p53
ATF3
APOPTOSIS
CHOP/GADD153
MCL-1
Bcl-2
PUMA/BBC3
Noxa…
RECOVERY
cyclinD1
CDK4…
StressorA
StressorB
apoptosis
ATF3
recovery
Trifluridine or
Vorinostat

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Appendix

Appendix Table 1. List of the common 73 differentially induced genes in 2ug/ml (24hrs) cisplatin treated parental (Calu6 and H23) compared to their cisR1 (Calu6cisR1, H23cisR1) sub-lines. Cut-off of q < 0.001. Bolded gene name represent genes in which there was a > log2 fold change in expression compared to the parental cell lines.

ABI2	CDC42BPA	**CREB3L1**	**HBA1**	LBD2	P4AH2	SEC24B	TSPAN14
AFAPIL2	**CDH10**	**CXCL1**	**HBA2**	**LPHN2**	**PCDH1**	**SECTM1**	**TSPAN8**
APOL1	**CEACAM1**	**CXCL6**	**HERC3**	**LSR**	PCDHB13	**SEMA3A**	**ZNF323**
ASE1	**CFB**	**EFS**	HPSE	**MAGEA3**	**PCSK1**	**SERPINB2**	
ATF3	CHRNAI	ETV1	IFT74	**MAGEA6**	**PDGFC**	SPRY2	
BCAS1	**CHRNA9**	**FAM133A**	**IL18**	**MEOX1**	PLAC8	STAT1	
BMP4	CKMT1B	**FAM26E**	**IL32**	NF2	**PLCH2**	**STX1A**	
CA8	**COL3A1**	**FOSB**	KIF1A	**NR2F1**	**RAB17**	**TBX18**	
CACNB4	**COL6A3**	**GDAP1L1**	**KRBOX1**	**NR5A2**	**SCG3**	TGM2	
CALHM2	**CORO6**	**GLRB**	LASP1	**NTS**	**SCRT1**	**TNFRSF19**	

Upregulated Downregulated

Appendix Table 2. List of the top 5 out of the 75 differentially induced genes in 2ug/ml (24hrs) cisplatin treated parental (Calu6 and H23) compared to their cisR1 (Calu6cisR1, H23cisR1) sub-lines. Ranked based on q-value significant changes as determined by cufflinks analyses.

Gene	Cell Line	cisR1	parental	Log2Δ	p_value	q_value
ATF3	H23	46.4291	264.445	2.50987	>1E-20	>1E-20
ATF3	Calu6	13.7497	119.337	3.11757	2.07E-11	4.13E-09
FOSB	H23	0.6919	8.4531	3.61069	>1E-20	>1E-20
FOSB	Calu6	1.0636	8.9552	3.0737	7.04E-14	2.65E-11
FAM26E	Calu6	4.217	0.0307	-7.09903	4.00E-15	2.23E-12
FAM26E	H23	0.613	0.0259	-4.56203	1.47E-08	9.96E-07
ZNF323	H23	18.5444	4.6025	-2.01047	3.06E-12	4.96E-10
ZNF323	Calu6	36.7349	8.858	-2.05209	5.48E-10	7.42E-08
CFB	Calu6	1.2418	13.6717	3.46059	4.20E-10	5.78E-08
CFB	H23	0.9159	3.838	2.06706	8.46E-05	0.001539

Appendix Table 3. GO Terms for Gene Enrichment Analysis of the 75 differentially induced genes

Homo sapiens (REF) GO biological process complete	#	#	Expected	Fold Enrichment	+/-	Raw P value	FDR
regulation of signal transduction	3113	25	9.62	2.6	+	2.74E-06	1.44E-02
↳ regulation of response to stimulus	4178	29	12.91	2.25	+	6.07E-06	1.91E-02
...gnal transduction	4799	32	14.82	2.16	+	3.88E-06	1.52E-02
↳ signaling	5120	36	15.82	2.28	+	9.86E-08	1.55E-03
↳ cell communication	5237	36	16.18	2.23	+	1.66E-07	1.31E-03

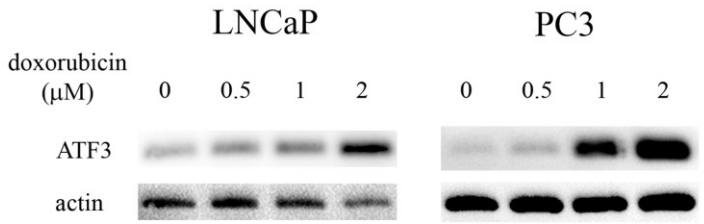

Figure 20: Western blot analysis of ATF3 induction by doxorubicin in prostate cancer cell lines LNCaP and PC3 following 24 hour treatment.

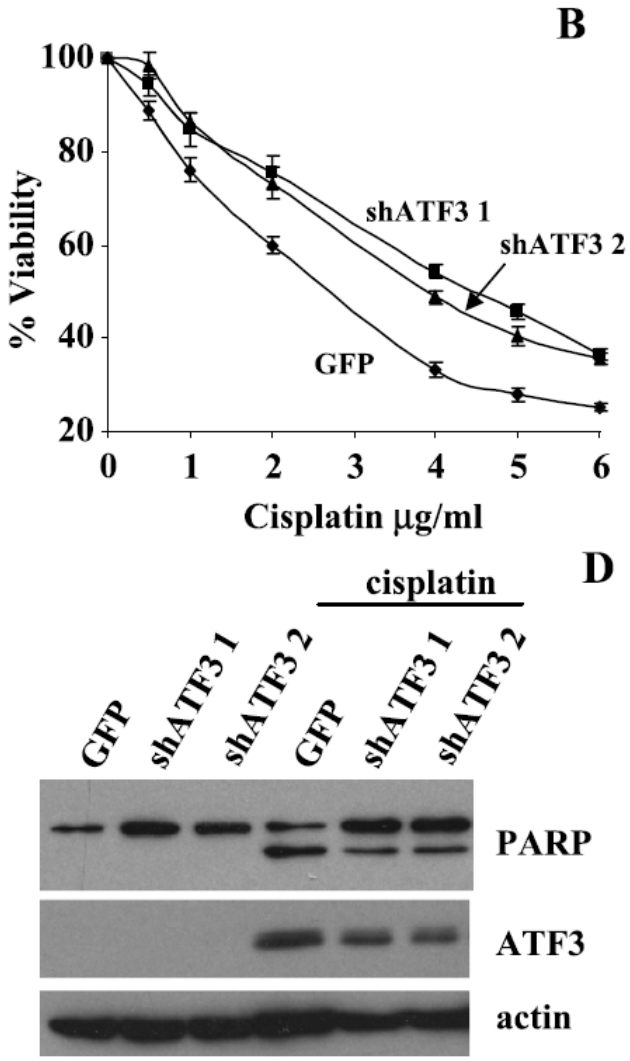

Figure 21. B) A549 cells stably expressing shRNAs targeting ATF3 mRNA (shATF3-1 and shATF3-2) or GFP (shGFP) as a control. MTT assay was performed following 48 hour treatment with cisplatin. D) Western blot analysis following 24 hour treatment with 10ug/mL cisplatin for A549 cells expressing shGFP, shATF3-1, and shATF3-2. From St. Germain et al. *Neoplasia* 2010 [209].

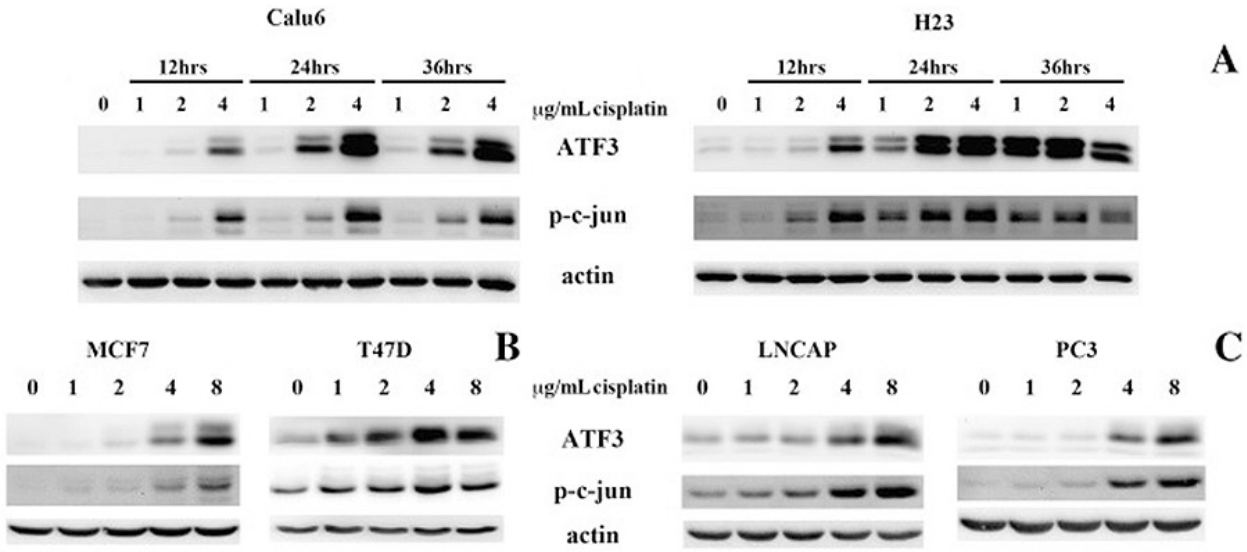

Figure 22: A) Western blot at 12, 24, and 36 hours for ATF3 and p-cjun expression following treatment with 1, 2, and 4 ug/ml cisplatin. B) and C) Western blot analysis for ATF3 and p-cjun following 24 hour treatment with cisplatin in breast cancer cells MCF7 and T47D, and prostate cancer cell lines LNCaP and PC3.

Curriculum Vitae

MOHAMED SHAAD HASIM

EDUCATION

- Doctor of Philosophy in Biochemistry 2013-Present
  University of Ottawa
  Thesis: The Role of Activating Transcription Factor 3 as a Regulator of DNA-Damaging Chemotherapy Cytotoxicity

- Master of Science in Cellular and Molecular Medicine 2010-2012
  University of Ottawa
  Thesis: Molecular mechanisms of MMP9 expression in astrocytes induced by heme and iron

- Honours Bachelor of Science 2006-2010
  Major in Biochemistry, Minor in Chemistry
  University of Ottawa

SKILLS

- Laboratory skills in biochemistry, chemistry, and biology
- Excellent interpersonal, communication and organizational skills
- Technical skills in Microsoft Word, Excel, and PowerPoint applications and internet research tools

PUBLICATIONS

1. Tam RY, Yockell-Lelievre J, Smith LJ, Julian LM, Choey C, Baker AEG, **Hasim MS**, Dimitroulakos J, Stanford WL, Shoichet MS. Rationally Designed 3D Hydrogels Model Invasive Lung Diseases Enabling High-Content Drug Screening. Adv Mater. 2018 Dec 27:e1806214.

2. **Hasim MS**, Nessim C, Villeneuve PJ, Vanderhyden BC and Dimitroulakos J. Activating Transcription Factor 3 as a Novel Regulator of Chemotherapy Response in Breast Cancer. Transl Oncol. 2018 Aug;11(4):988-998.

3. Bar J*, **Hasim MS***, Baghai T*, Niknejad N, Perkins TJ, Stewart DJ, Sekhon HS, Villeneuve PJ, Dimitroulakos J. Induction of Activating Transcription Factor 3 is associated with cisplatin

responsiveness in NSCLC: A potential predictive biomarker of response. Neoplasia. 2016 Sep;18(9):525-35. [* equal contribution]

4. Howe GA, Xiao B, Zhao H, Al-Zahrani KN, **Hasim MS**, Villeneuve J, Sekhon HS, Goss G, Sabourin L, Dimitroulakos J, Addison CL. Enhanced anti-tumor activity of erlotinib in combination with focal adhesion kinase inhibitors in non-small cell lung cancer. PLoS One. 2016 Mar 10;11(3):e0150567.

5. Bamji-Mirza M, Callaghan D, Najem D, Shen S, **Hasim MS**, Yang Z, Zhang W. Stimulation of insulin signaling and inhibition of JNK-AP1 activation protect cells from amyloid-β-induced signaling dysregulation and inflammatory response. J Alzheimers Dis. 2014;40(1):105-22.

WORK EXPERIENCE

Research Student (Doctorate)

University of Ottawa/Cancer Therapeutics Program, Ottawa Hospital Research Institute
2013- Present

Under the supervision of Dr. Jim Dimitroulakos

- Performed various biochemistry and molecular biology based experiments in order to identify and characterize novel therapeutic approaches for the treatment of cancer
- Contributed to the publication of our findings in peer-reviewed scientific journals
- Collaborated with other research groups on joint research projects
    - Was part of a team of surgeons, pathologists and researchers to obtain and perform experiments on human tumor specimens *ex-vivo*
- Worked closely with undergraduate students to train them on laboratory techniques including Western blotting, qPCR, cell culture, and more; helped guide them with their research projects

Research Student (Master's)

University of Ottawa/Institute of Biological Sciences, National Research Council 2010-2012

Under the supervision of Dr. Wandong Zhang

- Performed various biochemistry and molecular biology based experiments in order to characterize mechanisms of blood-brain barrier permeability following stroke
- Learned and used various laboratory techniques including cell culture, Western blotting, qPCR, and EMSA.
- Presented scientific findings at the Society of Neuroscience international meeting in 2011
- Contributed to the publication of our findings in peer-reviewed scientific journals
- Awarded Faculty of Graduate and Postdoctoral Studies Dean's Scholarship

Research Student (part-time Sept. to Apr. full-time May to Aug.)
2008- 2010

Cardiovascular Devices Division, University of Ottawa Heart Institute, Ontario

Under the supervision of Dr. Tofy Mussivand

- Worked with research team to draft patents for novel medical devices
- Patent preparation including researching literature and writing preliminary draft of patent application
- Trusted to work with confidential information

PRESENTATIONS

- M.S Hasim, C Nessim, PJ Villeneuve, J Dimitroulakos. The Role of ATF3 in Mediating Chemotherapy Response in Breast Cancer. OHRI Research Day 2016 **Poster**
- M.S Hasim, C Nessim, PJ Villeneuve, J Dimitroulakos. ATF3 as a Novel Regulator of Cisplatin Response in NSCLC. OHRI Research Day 2013 **Poster**
- M.S Hasim and Wandong Zhang. Expression and regulation of MMP-9 in rat astrocytes. Department of Pathology and Laboratory Medicine Annual Research Day 2012, University of Ottawa. **Poster**
- M.S Hasim and Wandong Zhang. Expression and regulation of MMP-9 in rat astrocytes. Society of Neuroscience Conference 2011, Washington D.C. **Poster**

AWARDS

Faculty of Graduate and Postdoctoral Studies Dean's Scholarship 2013

University of Ottawa Admission Scholarship 2006- 2007