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Enchancing the Efficacy of Platinum-Based Chemotherapeutics by Targeting BRCA1

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Enhancing the efficacy of platinum-based chemotherapeutics by targeting BRCA1

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master's of Science

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And last but not least, to my Uncle Steve, in whose memory I dedicate all that I have done and ever will do to contribute to this field of research.

ABSTRACT

Decreased expression of Breast Cancer 1 protein BRCA1 in breast and epithelial ovarian cancer patients has been correlated with increased overall survival. The improved outcome of patients with BRCA1-deficient tumours has been linked to their impaired ability to repair DNA damage induced by chemotherapy, specifically platinum agents. Targeting the DNA repair protein BRCA1 using novel therapeutic approaches has the potential to enhance platinum chemosensitivity in breast and ovarian cancer. Human ovarian and breast cancer cell lines were treated for 24 or 48 hours with three standard chemotherapeutics alone or in combination with three small molecule inhibitors that target DNA repair activity. Treatment with a histone deacetylase inhibitor sensitized breast and ovarian cancer cell lines to platinum agents. The HDACi significantly decreased BRCA1 RNA and protein expression. The HDACi shows promise as a therapeutic agent in combination with platinum therapy for breast and ovarian cancer due to its ability to down-regulate BRCA1.

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LIST OF ABBREVIATIONS

3-ABA	3-aminobenzamide
Acetyl H4	acetylated histone 4
ATM	ataxia telangiectasia-mutated kinase
ATR	ATM- and Rad3-related kinase
BASC	BRCA1-associated surveillance complex
BRCA1	breast cancer 1 protein
BRCT	BRCA1 C-terminal domain
CDDP	cisplatin
CDKs	cyclin-dependent kinases
Cdk2	cyclin-dependent kinase 2
ChIP	chromatin immunoprecipitation
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double strand break
EOC	epithelial ovarian cancer
ERCC1	excision repair cross-complementation group 1 protein
ERE	oestrogen responsive element
HAT	histone acetyltransferase
HDAC	histone deacetylase
HR	homologous recombination
HRP	horseradish peroxidase
IR	ionizing radiation
KRAS	Kirsten Ras
MDP2	methyl binding domain protein 2
MMR	mismatch repair
MRN	Mre11-RAD50-NBS complex
MTA1	metastasis-associated tumour antigen 1
MTT	methylthiazolyldiphenyl-tetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide
NBS1	Nijmegen breakage syndrome 1
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
NuRD	nucleosome remodelling and deacetylating
OS	overall survival
PARP1	poly (ADP-ribose) polymerase 1
PBS	phosphate-buffered saline
PARP1	poly (ADP-ribose) polymerase 1
PRR	positive regulatory region
RD	residual disease
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAHA	suberoylanilide hydroxamic acid
TBS-T	Tris-buffered saline with Tween-20
TSA	trichostatin A
XPF	xeroderma pigmentosum complementation group F protein

1. INTRODUCTION

1.1 Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) makes up more than 90% of all cancers arising from the ovary and is the fifth leading cause of cancer deaths in women behind breast, lung, colon, and stomach cancers (1, 2). This disease primarily affects women between the ages of 60 and 65 years (3). It is the most lethal of gynaecological cancers in North America, with a five-year survival rate of just 30%, due to the lack of effective screening methods and the absence of symptoms at early stages of the disease (1). Roughly 20% of patients are diagnosed with Stage 1 disease when the cancer is confined to the ovaries (1, 2, 3). Advanced stage disease indicates the cancer has metastasized to pelvic organs beyond the ovaries (Stage 2), beyond the pelvic organs to the abdomen (Stage 3), or beyond the peritoneal cavity (Stage 4) (1, 2). Roughly 70% of EOC cases are diagnosed at Stage 3 metastatic disease or higher (1, 2). Metastatic disease is accompanied by nonspecific symptoms such as gastrointestinal upset, anorexia, and abdominal distension, which are also shared by several common gastrointestinal, gynaecological, and genitourinary problems (2). The survival rate of EOC patients improves drastically to 90% if the disease is diagnosed at Stage 1 (3). Disease stage is, therefore, the most important prognostic factor in the management of EOC (1).

The pathogenesis and evolution of EOC is not well understood but malignant transformation is thought to be caused by genetic, molecular, and hormonal alterations that disrupt cell proliferation, apoptosis, senescence, and DNA repair mechanisms (4). Alterations of the tumour suppressor genes p53 and the Breast

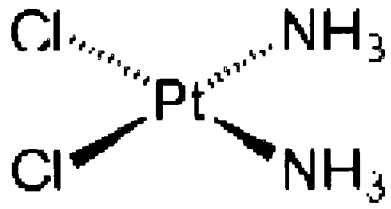
Cancer 1 protein (BRCA1) are known to play significant roles in the pathogenesis of this disease (4).

1.2 Treatment of EOC

Standard first-line treatment of EOC involves a combination of cytoreductive surgery and platinum- and taxane-based chemotherapy (1). Cisplatin [cis-diammine-dichloroplatinum(II)] was initially discovered to have growth-inhibitory effects in *Escherichia coli* in 1970 and it has been employed in the clinic for over 30 years (Figure 1A) (5, 6). The cisplatin analog, carboplatin [1,1-cyclobutanedicarboxylatodiamine-platinum(II)] shares a common mechanism of action and forms identical DNA lesions but contains a dicarboxylate leaving group as opposed to cisplatin's more labile chloride leaving groups (Figure 1A,1B) (6). Cisplatin and carboplatin are the most commonly used platinum agents in the clinic, used for the treatment of several types of cancer other than EOC, including testicular, cervical, head and neck, lymphoma, and non-small cell lung carcinomas (6). There are two less common platinum analogs, oxaliplatin [*trans*-(1,2-diammino-cyclohexane) platinum(II)] and satraplatin [bis(aceto)amminedichloro-(cyclohexyl-amine) platinum(IV)], approved for clinical use, however these drugs act through different mechanisms of action and elicit separate detoxification mechanisms and will not be discussed herein (6).

Cisplatin and carboplatin are synthetic DNA crosslinking agents that are aquated upon entering cells, a process that removes the chloride or dicarboxylate leaving groups from the molecules. While the resulting positively charged molecule can interact with nucleophilic molecules contained within the cell such as RNA,

A



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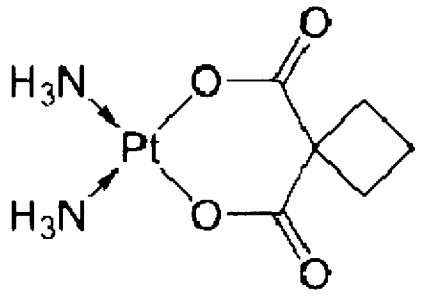

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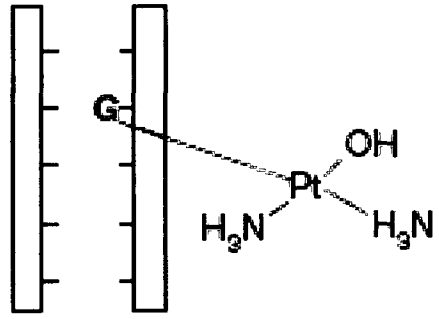
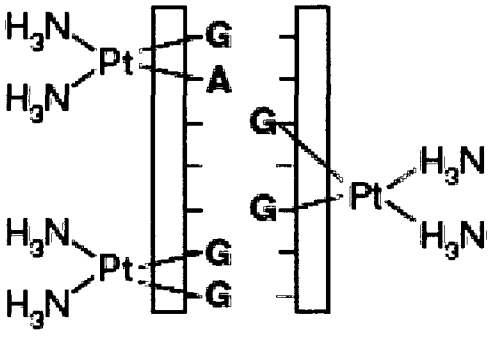
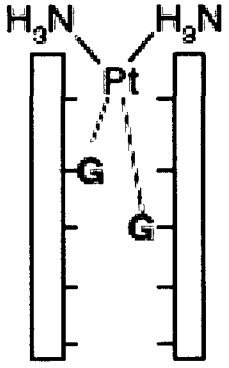


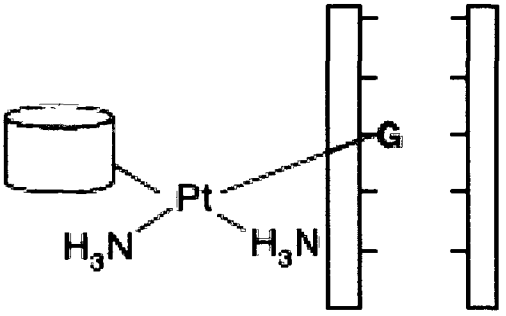
DNA Monoadduct



Intrastrand Crosslinks



Interstrand Crosslink



DNA-Protein Crosslink

Figure 1: Chemical structures and platinum DNA adducts. A. The chemical structure of the clinically relevant platinum agent cisplatin. B. The chemical structure of the clinically relevant platinum agent carboplatin. C. The primary DNA adducts created by platinum agents include monoadducts, interstand and intrastand crosslinks, and DNA-protein crosslinks. (Adapted and manipulated from Rabik, C. A. and Dolan, M. E. Molecular mechanisms of resistance and toxicity associated with platinating agents. Cancer Treat Rev, *33*:9-23, 2007.)

DNA, and protein, they preferentially bind DNA at the N7 atom of the imidazole ring of guanosine and adenosine (6). Binding to DNA can cause DNA monoadducts, intrastrand, interstrand, and DNA-protein crosslinks (Figure 1C) (6). Double strand breaks (DSBs) are caused by the cell's attempt to repair DNA interstand crosslinks created by platinum agents, the accumulation of which contributes to the cytotoxicity of these drugs (7). The majority (90%) of crosslinks formed are 1, 2 intrastrand d(GpG) adducts. This chelation causes a 40 - 70° kink in the DNA helix, effectively inhibiting DNA replication and transcription which is the primary cause of platinum cytotoxicity (6). By forming DNA-protein crosslinks, these compounds can also uncouple oxidative phosphorylation by the inhibition of mitochondrial ATPases (6, 8). Treatment with cisplatin can cause serious nephrotoxicity due to the inhibition of renal Na^+/K^+ -activated and Mg^{2+}-activated ATPases (8). Nephrotoxicity is not a side effect of carboplatin, possibly due to the slower reactivity of its leaving groups; therefore carboplatin is more commonly used in combination with paclitaxel in conventional EOC treatment (1).

Paclitaxel (also known as taxol) is a member of the taxane family of chemotherapeutics. It was initially isolated in 1971 from the bark of the Pacific yew tree *Taxus brevifolia* (9). Paclitaxel was originally documented to have anti-mitotic properties by Schiff *et al* in 1979 (10). Its mechanism of action involves the promotion of tubulin polymerization and the stabilization of microtubules. This inhibits the break-down of microtubules required for cell division and eventually leads to cell death (10, 11). Paclitaxel is approved for treatment of solid tumours such as those of the breast, ovarian, lung, and head and neck(12). Approximately 75% of

EOC patients respond favourably to this initial treatment regimen of cytoreductive surgery, either before or after the combination regimen of platinum and taxane chemotherapeutics (1, 2).

1.3 DNA repair and resistance to platinum-based chemotherapeutics in EOC

The development of platinum resistance after disease recurrence is a major obstacle in the treatment of EOC. While three-quarters of patients respond to the first-line regimen, response to treatment decreases drastically with disease recurrence (1, 13). In addition, if the relapse occurs within 12 months of cytoreductive surgery, response to the same platinum and taxane chemotherapy regimen is approximately 70%. However, if the relapse occurs within only 6 months of the surgical procedure, response to this treatment drops to only 10-15% (1). The development of platinum resistance is multifactorial involving many cellular processes such as membrane transport, glutathione inactivation, increased tolerance to DNA damage, and increased DNA repair (6, 14). The primary factor contributing to the development of platinum resistance is believed to involve the acquired tolerance to platinum-induced DNA damage by increased function of critical DNA repair pathways and their components (6, 14, 15).

The two primary pathways used by the cell to repair DNA adducts caused by platinum agents are the nucleotide excision repair (NER) and mismatch repair (MMR) pathways (14). The NER pathway is highly conserved and functions by repairing lesions in the DNA strand leading to alteration of its helical structure and inhibition of DNA replication and transcription. To begin, the DNA lesion must be recognized and marked for repair. More than thirty proteins, including BRCA1,

converge to form a complex that unwinds the damaged portion of the helix (16). A dimer consisting of the excision repair cross-complementation group 1 (ERCC1) protein and the xeroderma pigmentosum complementation group F (XPF) excises the lesion (14). Lastly, the excised portion of the DNA is resynthesized by DNA polymerase (14, 16). Increased function of components within the NER pathway has been correlated to increased resistance to the effects of cisplatin and carboplatin (6, 14). Targeting components of the NER pathway may be a viable therapeutic option to sensitize platinum-resistant tumours by impairing the tumour cells' ability to remove DNA lesions.

The MMR pathway is a highly conserved, strand-specific DNA repair process and is thought to mediate cisplatin- and carboplatin-induced apoptosis (17-21). The MMR process is initiated by the recognition of a DNA lesion (mismatched or unmatched DNA base pairs, or insertion-deletion loops) by the Mut proteins. The complex of proteins that perform the excision has yet to be elucidated (14). The excised portion of DNA is then resynthesized by DNA polymerase. It has been determined that platinum complexes interfere with the normal function of MMR, resulting in the accumulation of incompletely-repaired lesions, eventually leading to apoptosis (14, 21, 22). However, if there is a deficiency in MMR the cells can continue to proliferate in the presence of the platinum-induced lesions by bypassing the lesions, preventing the aforementioned platinum-induced MMR lesions (14). Tumours deficient in a functional MMR pathway have been found to be 2 – 3 fold more resistant to platinum-based chemotherapeutics than those with a functional MMR pathway (23).

Platinum-induced interstrand DNA crosslinks are known to cause the accumulation of DSBs in rapidly proliferating tumour cells, inevitably resulting in apoptosis (7). The cell has two complementary pathways to repair DSBs: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Figure 2) (4, 24). The HR repair pathway is considered to be an error-free method of repairing DSBs because the presence of sister chromatids is required for the accurate repair of the lesion (4, 24, 25). To initiate the HR process, the exposed ends of DNA at the site of the DSB are resected by a complex of DNA nucleases, Rad50/MRE11/Nijmegen breakage syndrome-1 (NBS1) in the 5' to 3' direction (24, 25). The resected ends are bound by Rad52, a DNA-end-binding protein, then Rad51 binds Rad52 creating a filament to facilitate the invasion of the double helix by a homologous sister chromatid. DNA polymerase extends the 3' tail using the information from the homologous section. The DNA crossovers (Holliday junctions) that result are resolved by DNA ligase to yield two homologous DNA double helices (24).

The process of NHEJ is accepted as an error-prone method of DSB repair as it does not use homologous sister chromatids to join the broken, sometimes slightly degraded ends of the DNA strands (4, 24, 25). The exposed termini are bound by the DNA-end-binding protein Ku, which recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The X-ray repair complementing defective repair in Chinese hamster cells 4 (Xrcc4) protein and DNA ligase IV are then recruited to the site to repair the lesion (24). The deficiency of genes involved in HR leads to the activation of the error-prone NHEJ pathway which creates genomic instability and

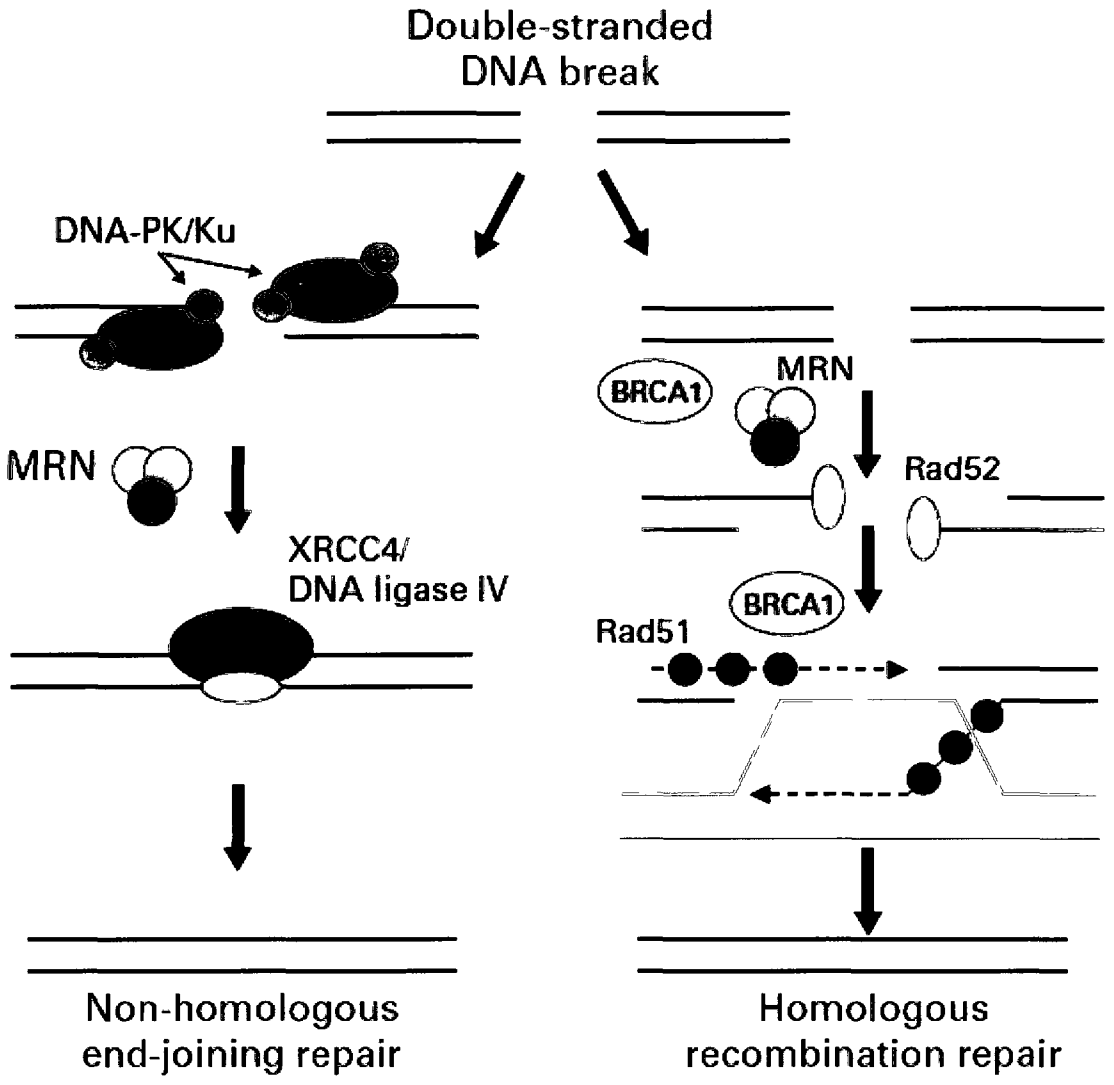

Double-stranded
DNA break
DNA-PK/Ku
MRN
XRCC4/
DNA ligase IV
Non-homologous
end-joining repair
BRCA1
MRN
Rad52
BRCA1
Rad51
Homologous
recombination repair

Figure 2: The cellular mechanisms of double strand break repair. The error-prone process of NHEJ directly links the severed DNA strands. The end-binding protein Ku recruits DNA-PK, XRCC4, and DNA ligase IV. Also believed to have a role in NHEJ is the Mre11-RAD50-NBS (MRN) complex. The error-free HR repair pathway involves the co-localization of BRCA1 and RAD51. RAD52 binds to the free DNA strands and RAD51 polymerizes to form a filament along which the broken 3' ends can invade the homologous duplex. To complete the repair, DNA polymerase extends the DNA strand. (Adapted from Weberpals, J. I., Clark-Knowles, K. V., and Vanderhyden, B. C. Sporadic epithelial ovarian cancer: relevance of the BRCA1 pathways and its function in DNA damage and repair. J Clin Oncol, *26*:3259 – 3267, 2008.)

cytotoxic stress, eventually leading to apoptosis (26). Hence, the increased expression or function of genes involved in the HR pathway may result in the tumour's ability to resist the cytotoxic effects of platinum chemotherapeutics. Therefore, an option to enhance sensitivity in platinum-resistant EOC may be targeting the HR machinery using novel therapeutics.

1.4 BRCA1

Of the oncogene and tumour suppressor genes that have been identified as contributors to the pathogenesis of EOC, including *P53*, Kirsten Ras (*KRAS*) and *c-MYC*, *BRCA1* is the most clinically relevant (4). *BRCA1* is a tumour suppressor whose loss through germ-line or somatic mutations can lead to the formation of breast and ovarian tumours. In approximately 10% of EOC, inherited germ-line mutations in the *BRCA1* gene play a role in tumour progression, while 90% of EOC cases occur sporadically (13). In sporadic EOC however, the BRCA1 protein is frequently dysfunctional, contributing to the pathogenesis of this disease (27, 28).

The *BRCA1* gene encodes a full-length protein of 1,863 amino acids with a molecular weight of approximately 220 kDa (29). This large protein includes several functional domains including the highly conserved NH_2 terminus containing a RING finger domain with E3 ligase activity and two nuclear localization signals in exon 11 (30, 31). The BRCA1 C-terminal domain (BRCT) from residues 1,646 to 1,863 contains a motif also present in proteins involved in DNA repair and genomic stability (32). This motif has been identified as a phosphopeptide recognition domain, binding phosphorylated proteins implicated in the DNA damage response (33, 34). The BRCT domain also interacts with histone deacetylases (HDACs) 1 and 2 leading

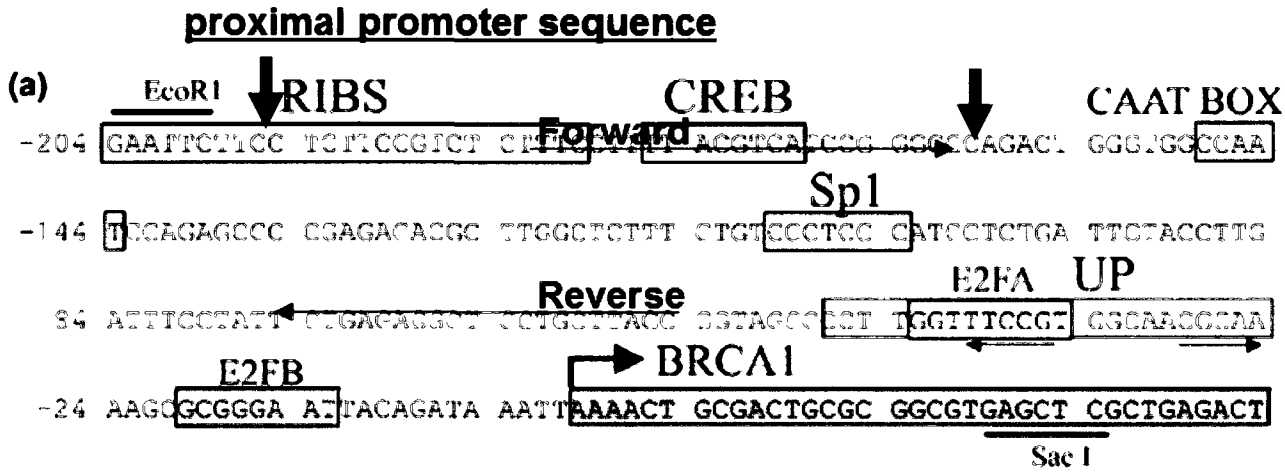
to repression of transcription, as well as the Rb-binding proteins, RbAp46 and RbAp48, implicating BRCA1 in growth inhibition through the binding of Rb (35). BRCA1 is known to interact with a variety of proteins in addition to those mentioned above, involved in a wide range of cellular activities such as cell cycle regulation, DNA damage response, stress response, apoptosis, transcriptional regulation, and chromosomal stability (36, 37). However, it is unknown how a single protein such as BRCA1 is involved in such a diverse group of cellular processes.

There is a large amount of information supporting the critical role of BRCA1 in the DNA damage response as supported by evidence indicating that the lack of a functional BRCA1 leads to the hypersensitivity of cells to various DNA damaging insults. BRCA1 is accepted to be involved in all aspects of DNA repair which include the recognition of damage, the prevention of replication during repair, and promotion or inhibition of apoptosis based on the level of damage present (25). The interaction between the BRCT and the HDACs indicates that BRCA1 may play a role in chromatin remodelling, which can partially explain the role of BRCA1 in the DNA damage response (35). In addition to this interaction, BRCA1 has been shown to be a component of the SWI/SNF chromatin remodelling complex and its coactivation function with p53 is mediated through this complex (38). The BRCA1-SWI/SNF complex may play a critical role in the activation of DNA damage response genes and may play a direct role in activating HR repair through chromatin remodelling by allowing the HR component access to sites of DNA damage (38). In fact, the aforementioned role of BRCA1 in the NER pathway can be explained by enabling the loosening of the chromatin structure, allowing for access of NER machinery to the

DNA lesion (25). In addition to NER, BRCA1 plays the role of a scaffolding protein which colocalizes at sites of DNA damage in the error-free DNA repair mechanism of HR and in MMR (25, 39-41). Its role as a scaffolding protein in both these DNA repair pathways originates from the involvement of BRCA1 in the genome surveillance repair complex called the BRCA1-associated surveillance complex (BASC). BASC is comprised of a number of proteins required for DNA damage signalling, recognition, and repair such as the ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) kinases, MMR-specific MLH1, MSH2 and MSH6 proteins and HR-specific proteins Rad50, MRE11, and NBS1 (25, 42). The recruitment of BASC and the resulting initiation of DNA damage response begin with the association of BRCA1 with Rad51 and BARD1 accumulating at sites of damaged and replicating DNA, in response to BRCA1 phosphorylation in the presence of DNA damaging agents (43). The hyperphosphorylation of BRCA1 and association with BARD1 and Rad1 recruits the components of BASC to the site of the DNA lesion (4). In HR, BRCA1 facilitates the activity of the 5'- to 3'-exonuclease complex (Rad50/MRE11/NBS1) and also, is known to associate to the Rad51 protein (25, 44). A process dependent upon the phosphorylation of BRCA1 by chk2 is the role of BRCA1 in promoting the error-free HR repair pathway over the error-prone NHEJ (25, 44-46). This indicates that functional BRCA1 is required for the maintenance of genomic stability through the prevention of the loss of genetic material through error-prone DNA repair mechanisms (25). The downregulation or loss of functional BRCA1 may lead to the disruption of functional DNA damage response, resulting in

genomic instability, cytotoxic stress, and dysfunctional DNA repair mechanisms due to its ubiquitous role in DNA damage.

Transcriptional control of *BRCA1* expression is complex and largely unexplored. In particular, signalling pathways which control negative regulation of *BRCA1* are unknown and there are several proposed mechanisms. Elucidating the mechanism of *BRCA1* downregulation is critical to determine the role of BRCA1 in predicting response to chemotherapeutics in EOC. *BRCA1* can be mapped to chromosome 17q21 and contains 24 exons (29). Exon 1 of the *BRCA1* gene contains an oestrogen responsive element (ERE) (47). The ERE is a positive regulatory element controlled by oestrogen stimulation. BRCA1 dysfunction is believed to results in tumorigenesis in breast and ovarian tissues because cell growth and proliferation in these tissue types is controlled by oestrogen stimulation (47, 48). The *BRCA1* promoter region is bidirectional, with a few hundred basepairs (bp) separating *BRCA1* from the *NBR2* locus (49, 50). Within the promoter, approximately 230 bp upstream from the transcriptional start site, is a highly conserved positive regulatory region (PRR) containing a RIBS element (Figure 3) (51, 52). It has been shown that the GA-binding protein α/β activates the transcription of *BRCA1* by binding to *ets* sites within the RIBS element (51). A CREB binding site is immediately downstream of the RIBS element and contained within the PRR (Figure 3) (52, 53). Further downstream, the promoter contains a CAAT box, Sp1 site, UP site which includes an E2FA site, and finally, an E2FB site immediately upstream of the transcriptional start site. The UP site and the E2F recognition site have been identified as repressor



BRCA1 **proximal promoter amplicon = 129 bp**

Figure 3: The *BRCA1* proximal promoter region. The sequence of the *BRCA1* proximal promoter region is shown with boxes indicating the various motifs, previously characterized. The sequence contained by the green arrows corresponds to the PRR. The black arrow shows the *BRCA1* transcriptional start site and the dark grey box following the black arrow indicates Exon 1 of the *BRCA1* gene. The red arrows represent the Forward and Reverse primers used in the ChIP assay protocol. The amplicon produced by these primers is 129 bp in length. (Adapted and manipulated from MacDonald, G., Stramwasser, M., and Mueller, C. R. Characterization of a negative transcriptional element in the *BRCA1* promoter. Breast Cancer Res, *9*: R49, 2007.)

elements (Figure 3) (52). A negative regulatory element was also located in the minimal *BRCA1* promoter contained within intron 1 of the gene (54), while a negative transcriptional repressor element identified within the first intron of *NBR2* was found to have no repressor activity on *BRCA1* (55). Interestingly, dynamic interactions between the promoter region of the *BRCA1* gene, the introns, and the terminator regions through gene loops have been reported to impact expression levels of the gene, in response to oestrogen stimulation and lactational development (48). Observations that the *BRCA1* promoter has a 56% cytosine and guanine content have led to the conclusion that the promoter contains CpG islands that can be aberrantly methylated causing gene repression by the binding of methyl binding domain protein 2 (MDP2) (56-59).

An increase in the expression of BRCA1 has been linked to platinum resistance in a wide range of cancers and is believed to be due to increased repair of DNA lesions (60-63). In addition, a recent study showed that in recurrent, platinum-resistant BRCA1-mutated ovarian tumours, genetic reversion had restored the wild-type reading frame leading to functional DNA repair in the tumour cells (64). Decreased expression of BRCA1 in EOC patients has recently been correlated with increased overall survival compared to those patients with increased expression (28, 60). This finding has been extended to include patients with breast cancer and non-small cell lung cancer, suggesting that BRCA1 function plays a significant role in the prognosis of a variety of malignancies (61, 65). Our group has found that higher BRCA1 expression was predictive of poorer overall survival (OS) in EOC patients

with residual disease (RD) following cytoreductive surgery less than 2 cm ($p = 0.03$) (Figure 4B) (13).

1.5 Targeting BRCA1 in combination with platinum chemotherapy

The improved outcome of patients with BRCA1-deficient tumours has been linked to the impaired ability to repair DNA damage induced by chemotherapy, specifically the platinum agents, leading to the accumulation of double strand breaks and eventually apoptosis (37). Genomic instability and cytotoxic stress have been observed in cells deficient in BRCA1 that have activated error-prone DNA repair pathways such as NHEJ (26). BRCA1-deficient mouse ovarian surface epithelial cells also show improved response to platinum agents (66). Our group has recently contributed to this field of evidence by showing that median OS was longer for EOC patients with lower BRCA1 expression as compared to those with higher BRCA1 expression (46 vs. 33 months, $p = 0.03$) (Figure 4A) (13). BRCA1 therefore, presents itself as an attractive therapeutic target to sensitize tumours to the cytotoxic effects of platinum chemotherapy in EOC as well as breast cancer. A number of novel small molecule inhibitors thus present themselves as rational candidates for combination with platinum chemotherapeutics because they may sensitize resistant tumours cells to the platinum agents by targeting DNA damage repair and perhaps more specifically, BRCA1 expression or function.

First, inhibitors of the poly (ADP-ribose) polymerase 1 (PARP1) enzyme are thought to potentiate the effects of DNA damaging agents given that PARP1 is an integral component of the DNA strand break repair machinery (Figure 5) (37, 67). As well, increased expression of PARP1 has been correlated to poorer outcome in

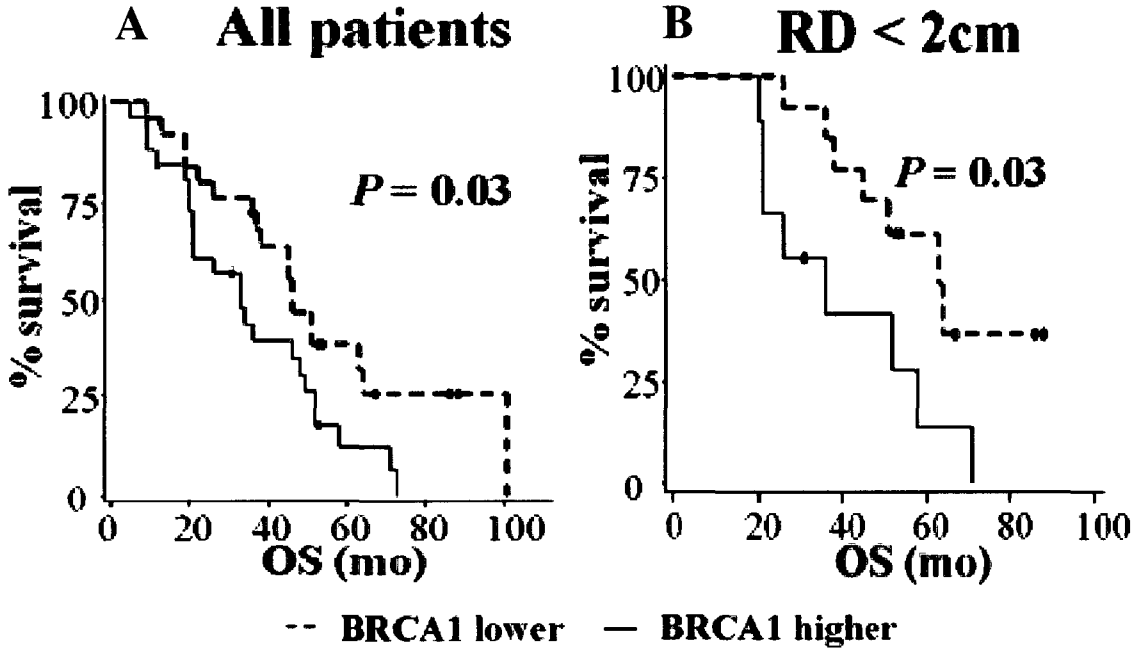

A All patients
B RD < 2cm
% survival
100
75
50
25
0
P = 0.03
0 20 40 60 80 100
OS (mo)
-- BRCA1 lower — BRCA1 higher

Figure 4: The predictive value of BRCA1 mRNA expression in EOC patients. BRCA1 mRNA levels expressed as a dichotomous variable comparing 51 patients divided in BRCA1-higher and BRCA1-lower groups. A. Overall survival (OS) for all patients divided into higher (represented by the solid line) and lower groups (represented by the dashed line). B. BRCA1-higher and BRCA1-lower groups compared in terms of OS for patients with residual disease (RD) <2cm (n = 22). (Adapted and manipulated from Weberpals, J. I., Garbuio, K., O'Brien, A., Clark-Knowles, K., Doucette, S., Antoniouk, O., Goss, G., and Dimitroulakos, J. The DNA repair proteins BRCA1 and ERCC1 as predictive markers in sporadic ovarian cancer. Int J Cancer, *124*:806-815, 2008.)

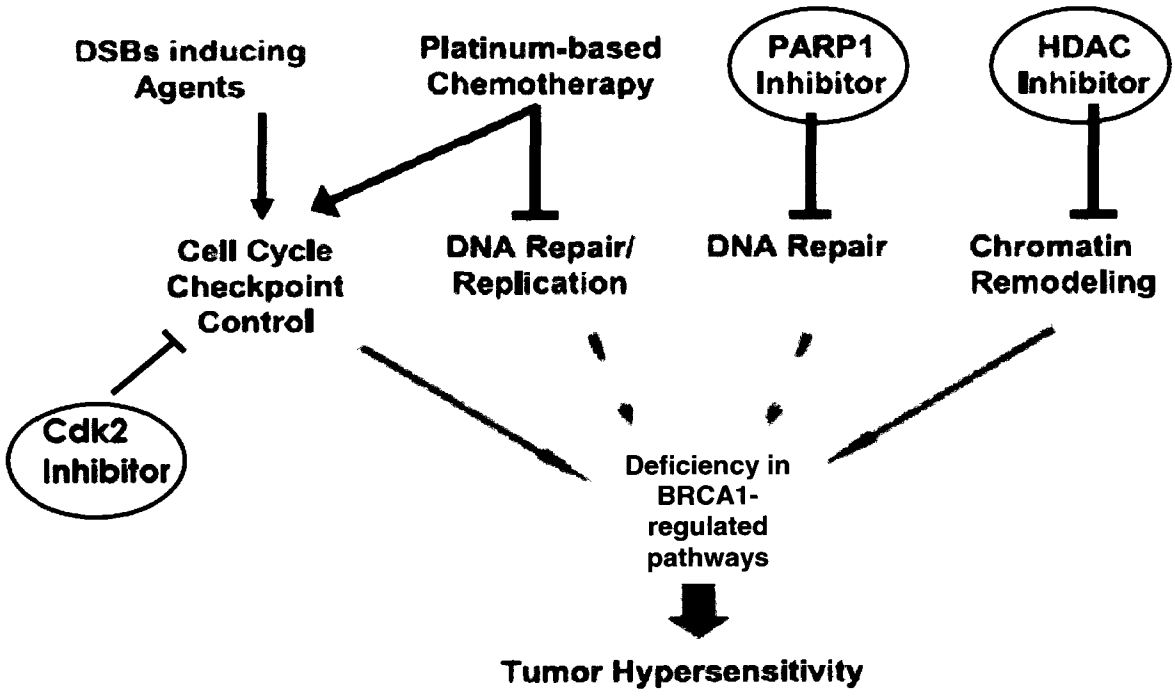

DSBs inducing Agents
Platinum-based Chemotherapy
PARP1 Inhibitor
HDAC Inhibitor
Cell Cycle Checkpoint Control
DNA Repair/ Replication
DNA Repair
Chromatin Remodeling
Cdk2 Inhibitor
Deficiency in BRCA1-regulated pathways
Tumor Hypersensitivity

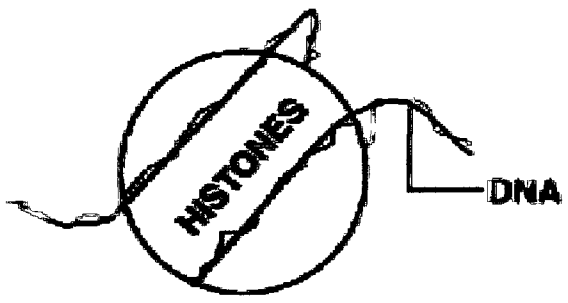
Figure 5: Novel small molecule inhibitors which may enhance platinum-induced cytotoxicity by targeting BRCA1-regulated pathways. The PARP1 inhibitor would interfere with normal DNA repair mechanisms, which would then become hypersensitive to DNA damaging agents. The Cdk2 inhibitor would prevent cell cycle checkpoint controls. HDAC inhibitors would render tumour cells sensitive to DNA damaging agents by interfering with chromatin remodelling. (Adapted and manipulated from Yarden, R. I. and Papa, M. Z. BRCA1 at the crossroad of multiple cellular pathways: approaches for therapeutic interventions. Mol Cancer Ther, *5*:1396-1404, 2006.)

ovarian cancer (68). PARP1 is a 113 kDa multifunctional, nuclear zinc-finger enzyme and has been documented to be involved in several cellular processes: DNA repair and maintenance of genomic stability, transcriptional regulation, regulation of cellular replication and differentiation, regulation of telomerase activity, activation of necrotic cell death, functional modification of enzymes by the addition of poly (ADP-ribose) polymers, and finally, the regulation of cytoskeletal organization (69). The enzymatic activity of PARP1 increases greater than 500-fold upon its binding to DNA single- and double-strand breaks (67, 69). PARP1 can detect DNA strand breaks caused by many agents, including reactive oxygen species (ROS), ionizing radiation (IR), and alkylating agents, as well as those strand breaks created during DNA repair processes (37, 67). Upon the detection of a DNA strand break, PARP1 catalyzes the transfer of ADP-ribose from co-enzyme nicotinamide adenine dinucleotide (NAD^+) to proteins involved in DNA metabolism and chromatin remodelling (67). Cells depleted of PARP1 *in vitro* showed increased cytotoxicity following treatment with DNA damaging agents, delayed DNA damage repair, and abnormal cellular morphology and chromatin structure (67). A deficiency in PARP1 causes the failure of the repair of single strand breaks. DSBs will form when the DNA replication fork in an actively dividing cell encounters an unrepaired single strand break (70, 71). In this study, inhibition of PARP1 by the compound 3-aminobenzamide, is anticipated to target BRCA1 function by interfering with the DNA repair machinery, thereby sensitizing tumour cells to DNA damaging agents, such as cisplatin and carboplatin.

The enzyme cyclin-dependent kinase 2 (Cdk2) induces cell cycle arrest to facilitate DNA repair before DNA replication occurs (Figure 5) (37, 72). Cyclin-

dependent kinases (CDKs) are a family of serine/threonine kinases required for cell cycle regulation and are activated by cyclins (72, 73). Specific inhibitors of CDKs are p21 and p27, which arrest cells in G_1 and G_2 cell cycle phases, respectively (73). It has been found that many human cancers, including those of the uterus and endometrium, possess deregulated CDK activity (74-77). Specifically, Cdk2 is not required for somatic cell division and its function in cell cycle regulation can be performed by other kinases. Cdk2 has several non-cell cycle-associated substrates which happen to function in DNA damage response, including BRCA1, BRCA2, p53, and Ku70, a NHEJ component (78-81). In fact, the inhibition of Cdk2 delayed the activation of the DNA damage response and prolonged the existence of DSB, effectively inhibiting DNA repair processes (72). A study by Deans *et al.* confirmed that Cdk2 can regulate both HR and NHEJ independently of cell cycle events (72). Their study found that Cdk2 inhibition delayed the activation of p53 and its target p21, as well as the phosphorylation of Chk1 following IR. This delay resulted in the reduction of Rad51 foci formation at sites of DNA damage (72, 82). Therefore, Cdk2 may act upstream of Rad51 and the DNA damage response as many of its targets are components in the protein complexes required for these pathways, including BRCA1 (72). Another study found that Cdk2 is present at chromosomal synapses during the exchange of genetic material between sister chromatids during prophase (83). BRCA1 is known to be involved in cell cycle checkpoints, and is activated by phosphorylation by Cdk2 (72); therefore, inhibiting Cdk2 using the purine analog 2(*bis*-(hydroxyethyl)amino)-6-(4-methoxybenzylamino)-9-isopropyl-purine, could mimic BRCA1 inhibition.

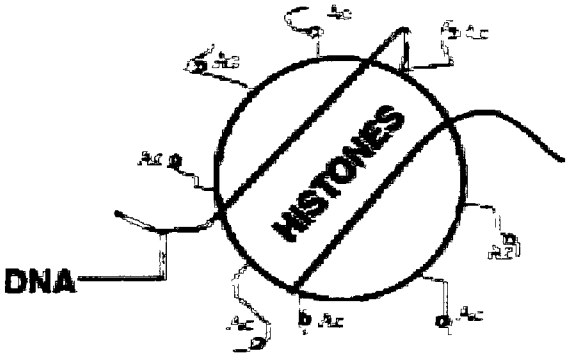
Finally, an emerging class of anticancer agents are the inhibitors of HDACs (Figure 5) (37) The interaction between histone acetyl transferase (HAT) and HDAC enzymes modulates chromatin structure and regulates transcription factor accessibility in order to modify gene expression (84) Histones contained by nucleosomes are acetylated by HATs and deacetylated by HDACs The substrate for these enzymes is a ε-amino group of lysine residues on the amino-terminal tails of the histones and the deacetylation of this lysine restores a positive charge to the residue which condenses the structure of the nucleosome (85, 86) Hence, silent genes are associated with nucleosomes with low levels of acetyled histones, whereas genes that are actively being transcribed are associated with nucleosomes with highly acetylated histones (Figure 6) (85, 87-89) Chromatin remodelling is particularly active during the DNA damage response, with rapid, localized unwinding of chromatin to allow for the entry and assembly of proteins that form DNA repair complexes (90) During this damage-induced remodelling HAT complexes work with ATP-dependent chromatin remodelling complexes to facilitate repair Chromatin structure also influences the susceptibility of a DNA strand to damage, with areas of loosely compacted, and hence more transcriptional active chromatin increasing the frequency of DSBs (90) HDAC inhibitors promote the accumulation of acetylated histones, and therefore, are thought to enhance platinum sensitivity by facilitating binding of DNA damaging agents to the unwound DNA strand (91, 92) There are four classes of HDAC enzymes and of these four classes, Class I enzymes have been found to be significantly overexpressed in ovarian tumour tissue versus normal ovarian tissue (93, 94) Intriguingly, when Class 1 HDACs are suppressed using siRNA, ovarian cancer cell growth is inhibited



Deacetylated Histones

↓

Transcriptional Repression



Hyperactylated Histone

↓

Transcriptional Activation

Figure 6: The mechanism of action of HDAC inhibitors. Upon treatment with HDAC inhibitors, histones become hyperacetylated (Ac) which loosens the DNA tightly wrapped around the histone. This loosening of the chromatin structure around acetylated histones allows transcriptional machinery access to the DNA which activates the transcription of target genes. HDACs function to deacetylate histones which would tighten the chromatin structure around the unacetylated histone, therefore repression transcription. (Adapted and manipulated from Marks, P. A., Richon, V. M., and Rifkind, R. A. Histone Deacetylase Inhibitors: Inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst, *92*:1210–1216, 2000.)

which supports the use of HDAC inhibitors as therapeutic agents in the treatment of EOC (93).

HDAC inhibitors have shown promise as anticancer agents, demonstrating inhibition of cancer cell growth *in vivo* and *in vitro* (95, 96). HDAC inhibitors are currently in Phase I and II clinical trials for advanced solid tumours and are approved for the treatment of cutaneous T-cell lymphoma (97-99). HDAC inhibitors have also demonstrated the ability to potentiate the effects of numerous chemotherapeutic agents in a pre-clinical setting, such as topoisomerase inhibitors, and DNA-damaging agents such as platinum compounds (92). It has been proposed that HDAC enzymes are important for HR repair of DSBs and the assembly of Rad51 subnuclear foci, of which BRCA1 is also a component (100, 101). Zhang *et al.* have shown that the HDAC inhibitor trichostatin A (TSA) delayed DNA damage response to IR by the suppression of key genes, including *BRCA1* (102). In addition, BRCA1 has been implicated in chromatin remodelling through its inclusion in the SWI/SNF chromatin remodelling complex, and interactions with HDAC isoforms HDAC1 and HDAC2, which are members of Class I HDAC group (35, 38, 94). HDAC inhibitors have shown potential as a therapeutic option in ovarian cancer and their ability to target BRCA1 makes them a very attractive option for this disease (Figure 5) (37, 93, 103, 104). In summary, an investigation into the effects of HDAC inhibitors on BRCA1 expression and function while in combination with platinum agents is warranted.

This study proposes that chemoresistance is regulated by proteins involved in key cellular pathways such as DNA damage response and repair, thus limiting the efficacy of platinum-based drugs. The overall objective of this study will be to

uncover therapeutic avenues to overcome chemoresistance in EOC and to provide the pre-clinical experimentation to justify clinical evaluation of this therapeutic approach. In addition, uncovering new factors regulating the expression of BRCA1 would make a significant impact on the breadth of knowledge concerning this crucial gene, as well as having wide implications on the lives of many cancer patients around the world.

Hypothesis: Targeting the critical DNA repair protein BRCA1 using novel therapeutic approaches will enhance the chemosensitivity of EOC cells.

Objectives:

1. Identify novel agents which enhance the cytotoxicity of platinum chemotherapeutics by targeting BRCA1-regulated pathways.
2. To investigate the mechanism of targeting BRCA1 expression by HDAC inhibitors.

2. MATERIALS AND METHODS

2.1 Cell Culture

The A2780s and A2780cp cell lines were kindly provided by Dr. B. Vanderhyden (Ottawa Hospital Research Institute, Ottawa, ON, Canada) and the T47D and OVCAR-4 cell lines were donated by Dr. J. Bell (Ottawa Hospital Research Institute, Ottawa, ON, Canada). MCF7 and HCC-1937 were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in Dulbecco's-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Wisent Inc., St-Bruno, Quebec, Canada) and 100 μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA). Unless otherwise described, cells were treated for 24 hours with 2 μg/ml cisplatin (provided by the pharmacy at the Ottawa Hospital Cancer Centre, Ottawa, Ontario, Canada) alone and in combination with the HDAC inhibitor M344 (Biovision, Mountain View, CA) at concentrations of 0.5, 1.0, or 5.0 μM. Phase contrast images were collected using the 10x objective of a Nikon Eclipse TE2000-U.

2.2 Cell Viability Assay

Cell viability was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) rapid colorimetric assay. Approximately 4,500 cells were seeded into each well of a 96-well flat bottom plate. The cells were incubated overnight to allow for cell attachment and recovery. Cells were then treated with cisplatin in concentrations of 0-8 μg/ml alone or in combination with 1 μM of the HDAC inhibitor M344, 50 μM of the PARP inhibitor, 3-aminobenzamide (3-ABA), or 5 μM of the Cdk2 inhibitor 2(*bis*-(Hydroxyethyl)amino)-6-(4-methoxybenzylamino)-9-isopropyl-purine (all from

Calbiochem, EMD Biosciences, Inc San Diego, CA) Forty-eight hours following treatment, 42 µl of a 5 mg/ml MTT substrate (Sigma-Aldrich, St-Louis, MO) solution in phosphate buffered saline (PBS) was added and incubated for up to 4 hrs at 37°C The resulting violet formazan precipitate was solubilized by the 82 µl addition of a 0 01M HCl/10% SDS (Sigma-Aldrich) solution and plates were incubated overnight at 37°C The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies (Chantilly, VA) at 570 nm to determine the optical density of the samples

2.3 RNA isolation and RT-PCR

Total RNA was extracted from cell lines in sub-confluent 10 cm dishes using the RNeasy® kit (Qiagen, Germantown, MD) RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc, Wilmington, DE) Total RNA (1 µg) was reverse transcribed for polymerase chain reaction (PCR) as previously described (105) The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification A real-time PCR reaction was carried out in a total volume of 25 µl that contained 2 5 µl of synthesized cDNA (42 ng), 1 25 µl of TaqMan Gene Expression Assay Primer/Probe (20X) (Applied Biosystems, BRCA1, HS00173233), 12 5 µl of TaqMan Universal PCR Master Mix (2X) (Applied Biosystems) and 8 75 µl of RNase-free water for BRCA1 expression GAPDH (Applied Biosystems, HS4333764-F) was used as an endogenous control Amplification conditions were 95°C for 5 min, 40 PCR cycles at 95°C for 15 sec, and 60°C for 1 min Three independent reactions from separate RNA extractions were used to determine the

average RNA expression and a standard error for each treatment condition. Technical assistance was provided by Anna O'Brien, research technician with Dr. Johanne Weberpals.

2.4 Western Blotting

Protein samples were collected in RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS]) containing 1X Protease Inhibitor Cocktail (Sigma-Aldrich) and protein content was quantified using a commercially available protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL) and a Biomate3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on 8-12% SDS polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA). Blocking was carried out with 5% milk in Tris-buffered saline with Tween-20 (TBS-T). For all subsequent immunoblotting, antibodies were diluted to the appropriate concentration in 5% milk in TBS-T. Blots were incubated with the following primary antibodies for 1 hour at room temperature or overnight at 4°C; mouse-anti BRCA1 (1:200, D-9, Santa Cruz, Santa Cruz, CA), rabbit-anti acetylated Histone 4 (acetyl H4) (1:1000, Upstate Cell Signaling now part of Millipore, Billerica, MA) and mouse-anti actin (1:5000, Sigma-Aldrich). Following three washes in TBS-T, blots were incubated with the appropriate horseradish peroxidase (HRP)-labeled secondary antibody (goat-anti-rabbit-HRP, goat-anti-mouse-HRP, 1:5000, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. The chemiluminescent substrate used was Supersignal West Pico (Pierce) and the visualization of the protein bands was performed using the GeneSnap image

acquisition system followed by densitometry analysis with the GeneTools software (Syngene, Frederick, MD).

2.5 Flow Cytometric Analysis of Apoptosis

Cells treated for 48 hours in 10 cm dishes were fixed in 80% ethanol for 1 hour. Cells were then washed with PBS and resuspended in staining buffer (0.2% Triton X-100, 1 mM EDTA in PBS pH 7.4) containing 25 μg/mL propidium iodide (Sigma-Aldrich) and 100 μg/mL RNaseA (Sigma-Aldrich). Cells were incubated with staining buffer in the dark for 1 hour prior to DNA quantification by the Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Data analysis was performed using ModFit *LT* (Verity Software House Inc., Topsham, ME).

2.6 Chromatin Immunoprecipitation (ChIP) Assay

Cells treated for 24 hours in 10 cm dishes were fixed with 1% formaldehyde (BDH, VWR International, Mississauga, ON) for 20 min at room temperature in order to cross-link the DNA and protein. The cross-linking was quenched by adding glycine to a final concentration of 200 mM and incubating at room temperature for 5 min. Cells were then washed twice with ice-cold PBS and harvested in 1 mL cold PBS by centrifugation at 4°C for 5 min at 5,000 rpm. The pellet was resuspended in 90 μL lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) supplemented with 1x Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM 1,4-dithio-DL-threitol (DTT) (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The lysates were sonicated using a Sonicator 3000 (Misonix, Farmingdale, NY) for a total of 3 min with 10 sec pulses and 10 sec on ice between pulses to shear DNA to an average size of 300 to 1000 bp. Sonicated lysates were cleared of debris

by centrifugation for 15 min at 14,000 rpm at 4°C. Input controls were removed from each sample and stored at -20°C. Sonicated lysates were divided into negative controls and positive samples, then diluted ten-fold with dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100) supplemented with 1x Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM DTT (Sigma-Aldrich), and 1 mM PMSF (Sigma-Aldrich). Positive sample cell lysates were immunoprecipitated by overnight rotation at 4°C with rabbit-anti acetyl H4 (1:200, Millipore) primary antibody. Negative controls were incubated overnight with rotation at 4°C in the absence of primary antibody.

Immune complexes were collected by 2 hour rotation at 4°C with the addition of 40 µL of protein A agarose/salmon sperm DNA 50% slurry (Millipore, Temecula, CA) to both positive samples and negative controls. The agarose beads/immune complexes were then pelleted gently by centrifugation for 1 min at 3,000 rpm at 4°C. The beads were washed with 1 mL of the following buffers by rotation for 10 mins at 4°C, then pelleted gently by centrifugation for 1 min at 3,000 rpm at 4°C, discarding the supernatant following each wash: Buffer A (low salt; 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 150 mM NaCl) once, Buffer B (high salt; 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 500 mM NaCl) once, Buffer C (1% NP-40, 1% sodium deoxycholate, 20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.25 M LiCl) once, TE washing buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) twice. Freshly prepared elution buffer (1% SDS, 100 mM $NaCHO_3$) was added to all samples (input and negative controls, and positive samples) to a final volume of 400 µL and samples were rotated at room

temperature for 30 mins. The agarose beads were removed from the samples by centrifugation for 1 min at 3,000 rpm.

The DNA-protein cross-linking was reversed by overnight incubation with 5 μL proteinase K (20 mg/mL, Roche Diagnostics, Laval, Que.) at 65°C. The DNA was purified using a QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was eluted in 50 μL ddH_2O and samples were stored at -80°C. Quantitative PCR was performed using a Roche LightCycler Version 3 (Roche Diagnostics). Amplification conditions were 95°C for 30 sec, 40 PCR cycles of 95°C for 1 sec, 57 °C for 10 sec, and 72 °C for 5 sec, and finally, 61°C for 40 sec. The binding of acetyl H4 to the BRCA1 proximal promoter region was determined using the following primer pair: forward TTTCCTTTTACGTCATCCGGG and reverse GCTAAGCAGCAGCCTCTCAGA (Figure 3) (52, 106). PCR products were resolved on 1.6% agarose gels.

2.7 Statistical Analysis

The probability of significant differences was determined by analysis of variance (ANOVA; multiple groups). Bonferroni's posttest was used to determine significance between specific treatments when whole group differences were detected by ANOVA. For all analyses, significance was inferred at $P<0.05$ and P values were two-sided. Analyses were performed using Graphpad Prism statistical software (Graphpad Software, San Diego, CA). Technical assistance was provided by Katherine Clark-Knowles.

3. RESULTS

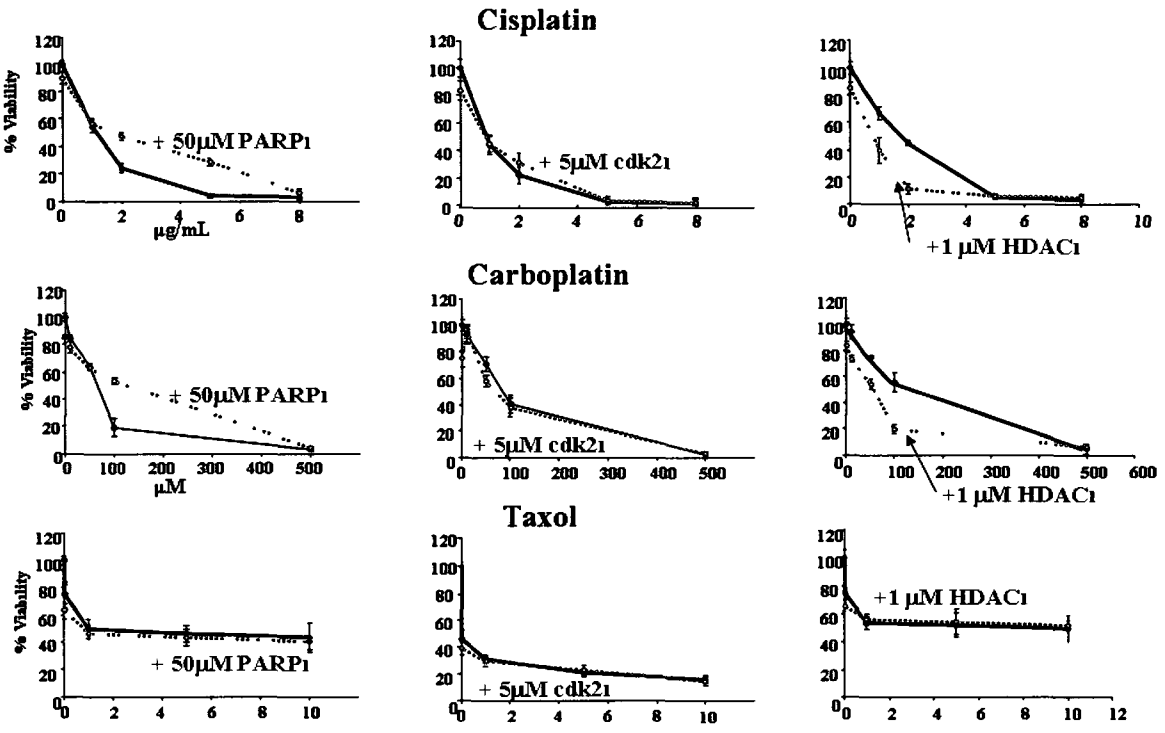
3.1 The inhibition of HDACs enhances platinum cytotoxicity in an ovarian tumour cell line and its platinum-resistant clone *in vitro*.

The ability of small molecule inhibitors of PARP, Cdk2, and HDAC (see Section 1.5) to potentiate the cytotoxicity of platinum-based chemotherapeutics was evaluated *in vitro* in an ovarian tumour cell line A2780s using the MTT cell viability assay. There was no improvement in drug sensitivity when the PARP inhibitor and CDK2 inhibitor were added to chemotherapy drugs (cisplatin, carboplatin, and paclitaxel) (Figure 7). Only the HDAC inhibitor M344 significantly increased sensitivity of A2780s and A2780cp cells to cisplatin and carboplatin cytotoxicity. There was no effect of this inhibitor on the cytotoxicity of paclitaxel (Figure 7). This experiment was also performed in the cisplatin-resistant A2780s-derived cell line clone A2780cp, with similar results (data not shown).

3.2 The HDAC inhibitor M344 targets BRCA1 expression in an ovarian tumour cell line *in vitro*.

The mechanism of enhancement of cytotoxicity by combining small molecule inhibitors with platinum-based chemotherapy may involve targeting BRCA1 as targeting BRCA1 expression can potentiate the cytotoxic effects of cisplatin (37, 66). Quantitative, real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) was used to determine changes in BRCA1 expression at the transcriptional level in the A2780s cell line. The A2780s control treatment was used as the calibrator for the RT-PCR analysis of expression of mRNA. Upon exposure of the cell lines to

A.


Cisplatin
Carboplatin
Taxol
% Viability
+ 50μM PARPi
+ 5μM cdk2i
+1 μM HDACi
μg/mL
μM

B.

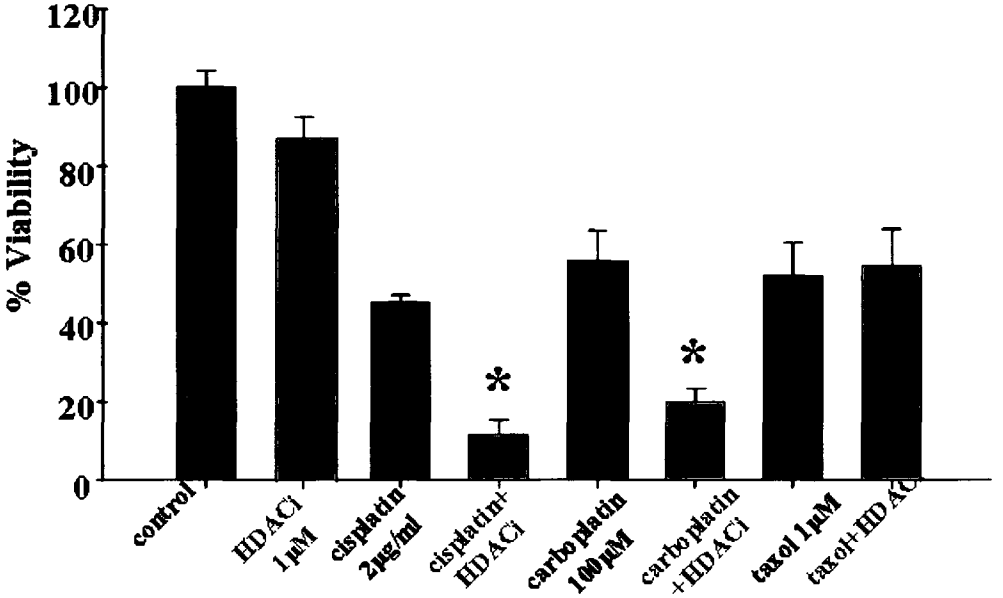

% Viability
control
HDACi 1μM
cisplatin 2μg/ml
cisplatin+ HDACi
carboplatin 100μM
carboplatin +HDACi
taxol 1μM
taxol+HDAC

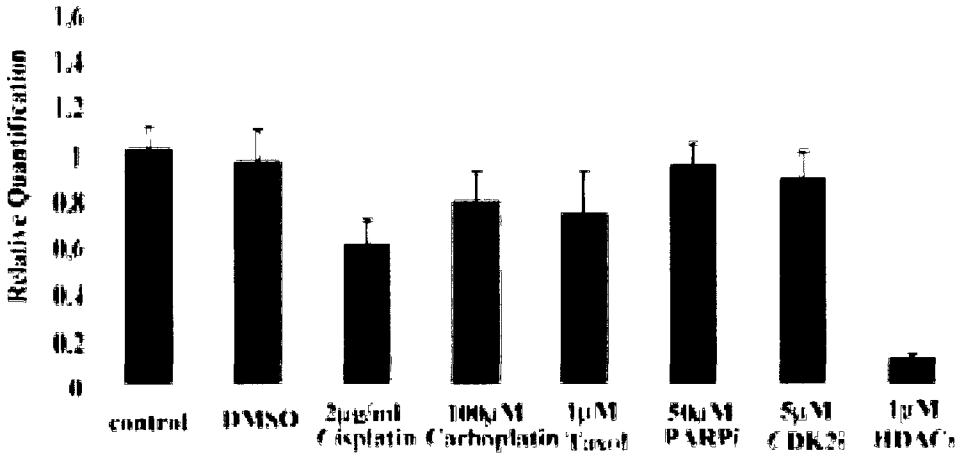
Figure 7: The inhibition of HDACs enhances platinum cytotoxicity in an ovarian tumour cell line and its platinum-resistant clone *in vitro*. A. MTT viability assays comparing the responses of 0–8 μg/mL cisplatin, 0–500 μM carboplatin or 0–10 μM taxol alone (represented by a black solid line) or with the co-administration (represented by a red dashed line) of 50 μM PARP1 inhibitor, 5 μM Cdk2 inhibitor or 1 μM HDAC inhibitor in A2780s cells. Cell viability was assayed with the activity of untreated cells taken to be 100%. B. *Combination of treatments in A2780s cells that displayed significant differences in MTT activity compared to either agent alone ($P < 0.01$, Paired T-test). MTTs were analyzed at 48 hr consistent with the methodology employed by the National Cancer Institute (107).

cisplatin, carboplatin, and paclitaxel, there were subtle changes in BRCA1 mRNA levels (Figure 8A). Treatment with the PARP and CDK2 inhibitors had relatively no effect on BRCA1 mRNA transcripts. Western blot analysis was employed to evaluate changes in BRCA1 protein expression in A2780s cells upon treatment with the HDAC inhibitor (Figure 8B). There was a significant decrease in mRNA and protein levels of BRCA1 after treatment with the HDAC inhibitor M344, suggesting that this compound can target DNA repair by modulating the expression of a key regulator of this pathway (Figure 8). The level of acetylation of histone 4 (acetyl-H4) was evaluated by western blot analysis as a positive control for the function of M344. The observed increase of acetyl-H4 following treatment with M344 indicates that this inhibitor is functional in these cells (Figure 8B). This experiment was also performed in the cisplatin-resistant A2780s-derived cell line clone A2780cp, with similar results (data not shown).

3.3 Basal expression of BRCA1 in a panel of breast and ovarian cancer cell lines.

This study was expanded to include three breast cancer cell lines (MCF7, T-47D, and HCC1937) and three ovarian cancer cell lines (A2780s, A2780cp, and OVCAR4) which were analyzed for basal BRCA1 expression via western blot analysis (Figure 9). The breast carcinoma line MCF7 was used as a positive control and was used as the calibrator for analysis of expression of protein levels and assigned an expression value of 1.0. MCF7 cells displayed the highest levels of BRCA1 protein of the breast cancer cell lines (Figure 9). The HCC1937 breast carcinoma cells did not display any detectable BRCA1 protein (Figure 9). HCC1937 cells harbour the germline BRCA1 mutation 5382insC, which results in a frameshift causing a premature stop codon and

A


Relative Quantification
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
DMSO
2μg/ml Cisplatin
100μM Carboplatin
1μM Taxol
50nM PARPi
5μM CDK2i
1μM HDACi

B

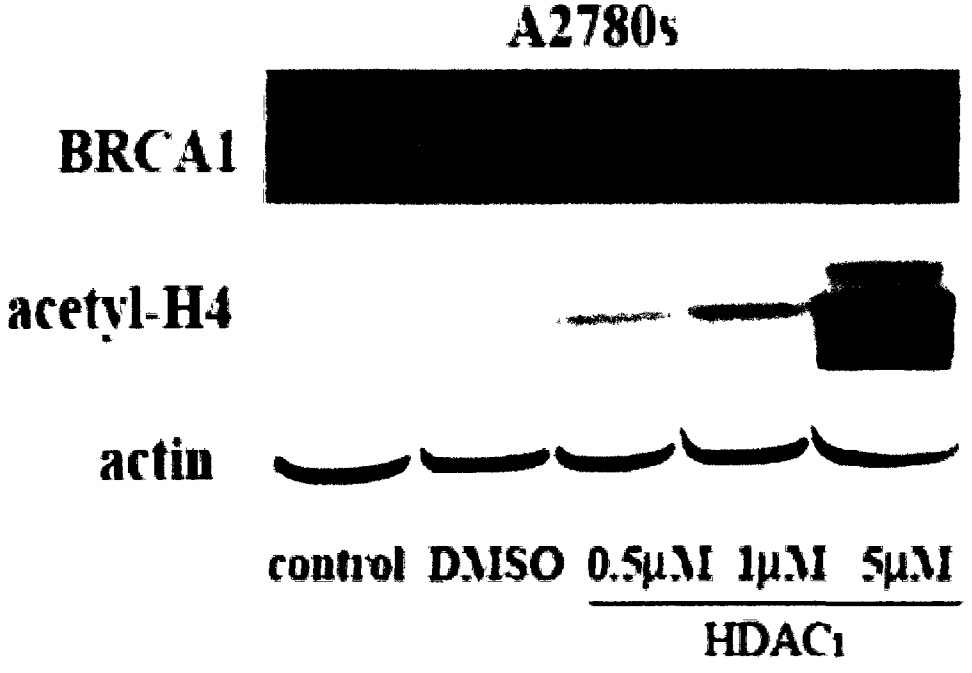

A2780s
BRCA1
acetyl-H4
actin
control
DMSO
0.5μM
1μM
5μM
HDAC1

Figure 8: The HDAC inhibitor M344 targets BRCA1 expression in an ovarian tumour cell line. A. Levels of BRCA1 mRNA analyzed by real time quantitative RT-PCR following 24 hr treatments as indicated. Fold changes were calculated following normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels ($\Delta\Delta$Ct) and expressed as means (+/- SEM). B. Levels of BRCA1, acetyl-H4, and actin protein as loading control, following 24 hr HDACi treatment in A2780s cells.

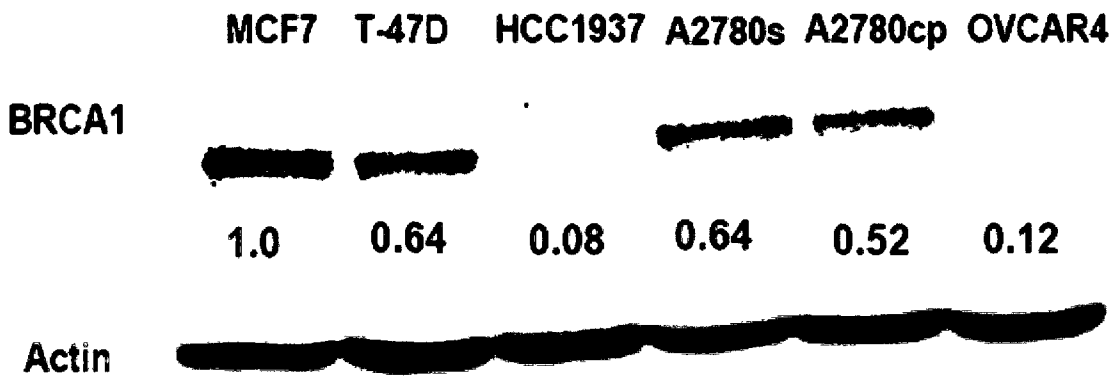
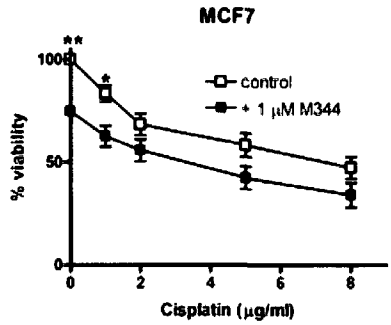

MCF7
T-47D
HCC1937
A2780s
A2780cp
OVCAR4
BRCA1
1.0
0.64
0.08
0.64
0.52
0.12
Actin

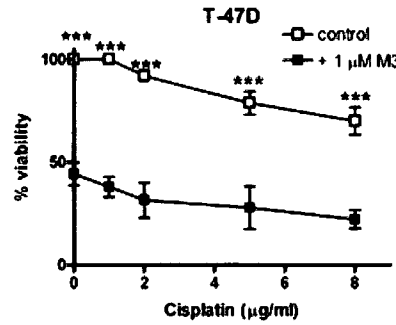
Figure 9: Basal expression of BRCA1 in a panel of breast and ovarian cancer cell lines. A. Western blot analysis of basal expression levels of BRCA1 protein in a panel of cell lines. Actin was used as a loading control. Numbers indicate protein densitometry readings with MCF7 used as the calibrator and set to 1.0.

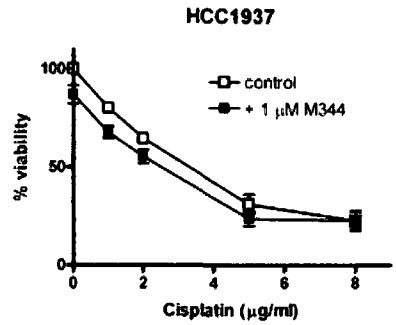
resulting in a truncated non-functional protein product (108). A2780s expressed the highest protein levels of the ovarian cancer cell lines, but only slightly more than their cisplatin-resistant counterpart A2780cp cells (Figure 9). There was a lack of detectable BRCA1 protein in the OVCAR4 cell line in addition to the HCC1937 cell line, despite its BRCA1 status being known as normal (Figure 9). This group of cell lines with variable levels of BRCA1 protein expression corresponds to the variation of BRCA1 protein expression levels seen in the clinical setting (13, 60-65).

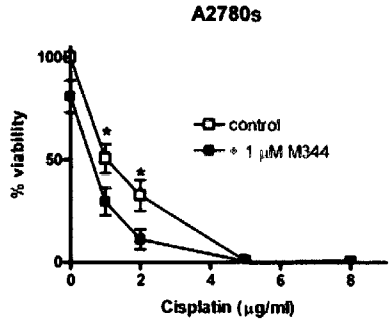
3.4 Treatment with the HDAC inhibitor M344 enhances the efficacy of cisplatin treatment in breast and ovarian cancer cell lines *in vitro*.

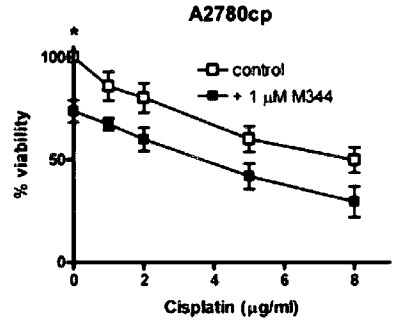
Effects on cell viability by treatment of M344 in combination with cisplatin on the six cell line panel were measured using the MTT cell viability assay. The addition of M344 to cisplatin did not enhance the effect of cisplatin in the HCC1937 breast cancer cells which have no detectable basal BRCA1 protein expression (see Section 3.3) (Figure 10). In MCF7 cells, cell viability was reduced with the addition of M344 to cisplatin treatment, but only at the lowest dose of cisplatin used (1 μg/ml, $P < 0.05$) was the effect statistically significant (Figure 10). Cell viability of the T-47D cells was significantly lower, by more than 50%, with the addition of M344 to cisplatin treatment at all the concentrations of cisplatin used ($P < 0.001$ at all concentrations) (Figure 10). Treatment with M344 enhanced the effect of cisplatin treatment in the A2780s cells at concentrations of 1 and 2 μg/ml cisplatin ($P < 0.05$) (Figure 10). No statistically significant effect was observed in the A2780cp cells, though M344 was observed to increase the cytotoxicity of cisplatin slightly (Figure 10). An enhancement of effect was only observed at a dose of 1 μg/ml cisplatin in the


MCF7
control
+ 1 μM M344
% viability
Cisplatin (μg/ml)


T-47D
control
+ 1 μM M344
% viability
Cisplatin (μg/ml)


HCC1937
control
+ 1 μM M344
% viability
Cisplatin (μg/ml)


A2780s
control
+ 1 μM M344
% viability
Cisplatin (μg/ml)


A2780cp
control
+ 1 μM M344
% viability
Cisplatin (μg/ml)

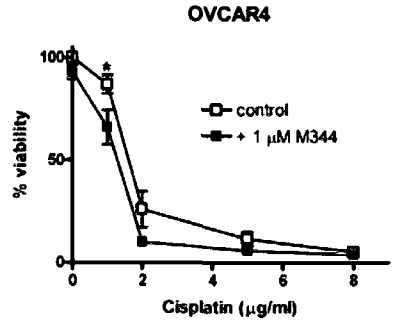
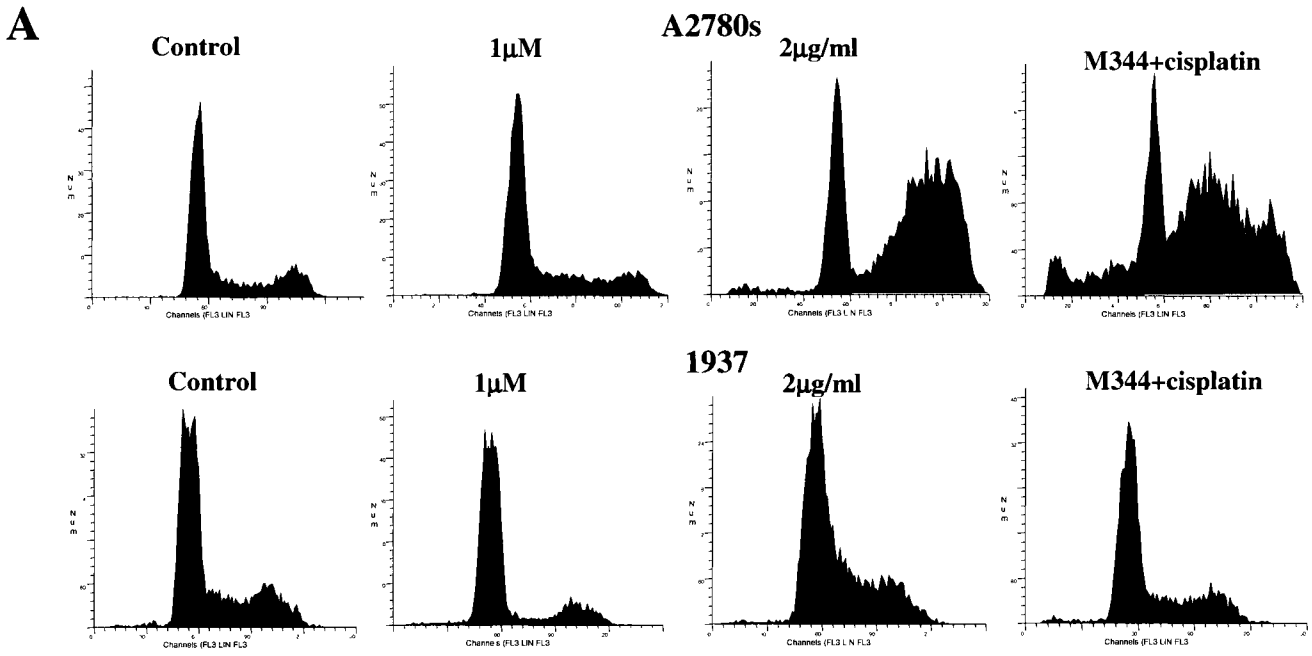

OVCAR4
control
+ 1 μM M344
% viability
Cisplatin (μg/ml)

Figure 10: Treatment with the HDAC inhibitor M344 enhances the efficacy of cisplatin treatment in breast and ovarian cancer cell lines *in vitro*. MTT viability assays comparing the responses of a group of cell lines to 0-8 μg/ml cisplatin alone (□) or with co-administration of 1 μM M344 (■). Cell viability was assayed with the activity of untreated cells taken to be 100%. Numbers represent the mean +/- SEM where n= 3 in triplicate. Differences between treatment with cisplatin alone versus treatment with cisplatin and M344 were analyzed using 2-way ANOVA with Bonferroni posttest. * indicates a significant difference where $P<0.05$, ** where $P<0.01$, and *** where $P<0.001$.

OVCAR4 ($P < 0.05$) ovarian cancer cells, which have no detectable basal expression of BRCA1 protein (refer to Section 3.3) (Figure 10). These results indicate that the HDAC inhibitor M344 showed a trend towards increasing platinum-induced cytotoxicity in this group of cell lines.

3.5 Treatment with the HDAC inhibitor M344 in combination with cisplatin increases apoptosis.

Cells from a representative breast cancer cell line (HCC1937) and a representative ovarian cancer cell line (A2780s) were treated with M344 and cisplatin, alone or in combination, for 48 or 24 hours respectively and then subjected to flow cytometric analysis to confirm that the cytotoxicity observed in the MTT cell viability assay was due to apoptosis. Treatment with M344 alone did not cause a dramatic increase in apoptosis versus untreated cells (Figure 11A). Treatment with cisplatin alone resulted in increased apoptosis in as compared to controls as shown by a slight increase in the sub-G1 population (Figure 11A). Treatment of the A2780s cells with a combination of M344 and cisplatin resulted in an increase in apoptosis levels above what was observed with either agent alone which corresponds to the results shown by the MTT cell viability assay in Section 3.4 (Figures 10 and 11A). There was no increase observed in the HCC1937 cell line, which also corresponds to the MTT cell viability assay results (Figures 10 and 11A). The A2780s cell line was too sensitive to cisplatin single-agent and M344 combination treatment to be evaluated for apoptosis by flow cytometry after 48 hours of treatment and thus was assessed after a 24 hour treatment. An increase in apoptosis in response to the cisplatin+M344 combination treatment was also seen by flow cytometry in the A2780cp and MCF7 cell lines (data not


A
A2780s
Control
1μM
2μg/ml
M344+cisplatin
1937
Control
1μM
2μg/ml
M344+cisplatin

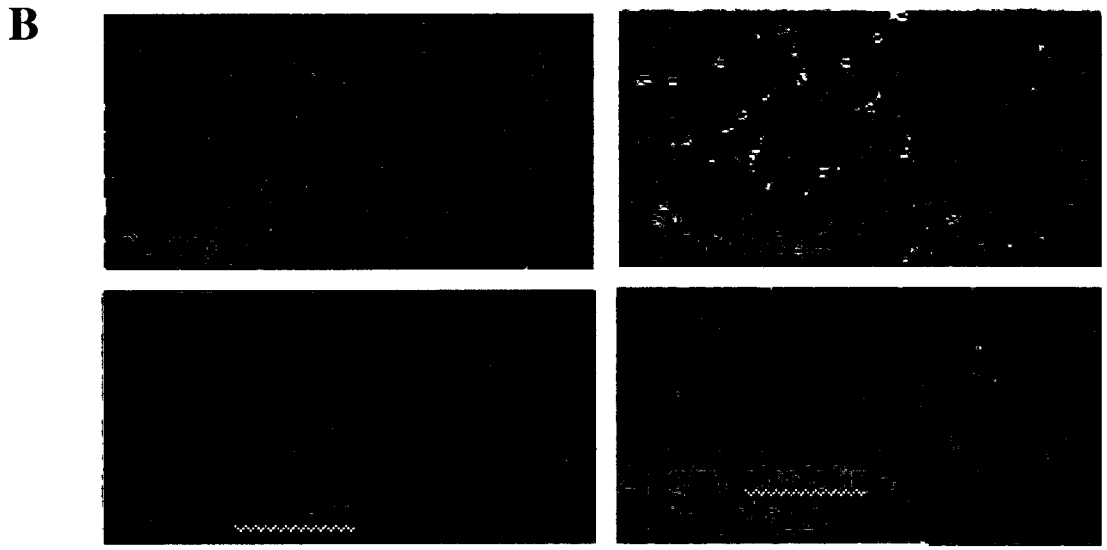
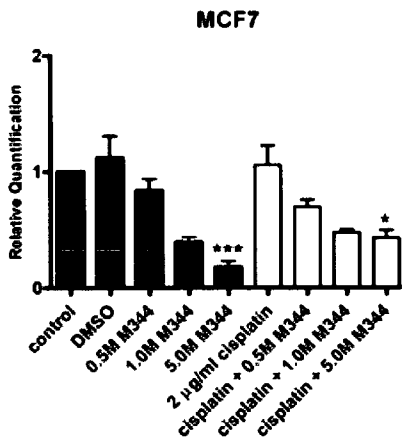

B

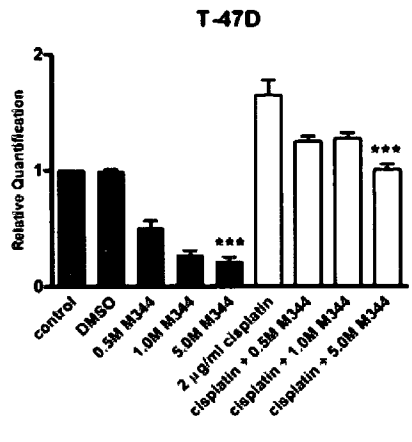
Figure 11: Treatment with the HDAC inhibitor M344 in combination with cisplatin increases apoptosis. A. Flow cytometric profiles of A2780s and HCC1937 following treatment with 1.0 μM M344 alone or in combination with 2 μg/ml cisplatin. B. 10x phase contrast images of A2780s cells following 24 hours of treatment with 1.0 μM M344 alone or in combination with 2 μg/ml cisplatin.

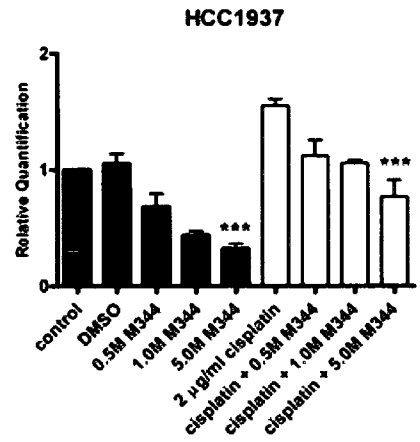
shown). The A2780s cells were used to obtain 10x phase contrast images following a 24 hour treatment period. These images show significant characteristics of apoptosis such as rounding up and lifting off of the culture plate in the cisplatin+M344 combination treatment (Figure 11B). The results from these experiments support that the cytotoxicity observed in the cisplatin+M344 combination treatment is due to the induction of apoptosis.

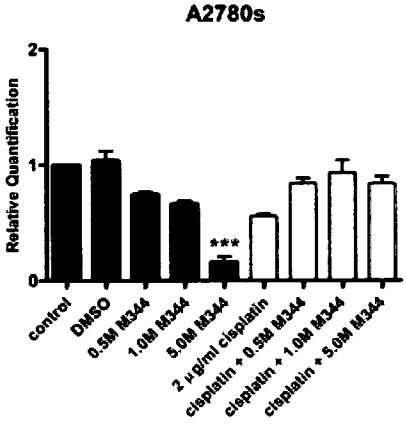
3.6 Treatment with the HDAC inhibitor M344 reduces BRCA1 protein and mRNA expression in breast and ovarian cancer cell lines.

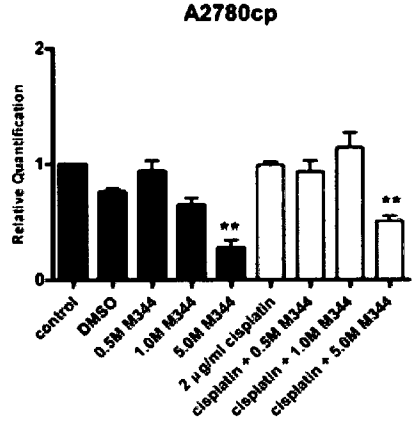
The mRNA and protein levels of BRCA1 were assessed following exposure to M344 alone or in combination with cisplatin by RT-PCR and western blot analysis, respectively. In the breast cancer cell lines examined, treatment with M344 alone resulted in a significant decrease in BRCA1 mRNA expression at the highest concentration as compared to DMSO-treated controls (Figure 12). With the exception of MCF7, BRCA1 mRNA levels were increased with cisplatin treatment alone as compared to untreated control in the two breast cancer cell lines (Figure 12). The addition of M344 to cisplatin treatment resulted in a decrease in BRCA1 mRNA levels in the two breast cancer lines (Figure 12). BRCA1 protein levels were found to decrease after treatment with M344 alone as compared to DMSO control and were also found to decrease after treatment with cisplatin in combination with M344 as compared to cisplatin alone in all the breast cancer cell lines examined (Figure 13). Due to their mutant BRCA1 status, BRCA1 protein was not detectable in the HCC1937 cells and thus protein levels in response to treatment were not assessed in these cells.


MCF7
Relative Quantification
2
1
0
***
*
control
DMSO
0.5M M344
1.0M M344
5.0M M344
2 μg/ml cisplatin
cisplatin + 0.5M M344
cisplatin + 1.0M M344
cisplatin + 5.0M M344


T-47D
Relative Quantification
2
1
0
***
***
control
DMSO
0.5M M344
1.0M M344
5.0M M344
2 μg/ml cisplatin
cisplatin + 0.5M M344
cisplatin + 1.0M M344
cisplatin + 5.0M M344


HCC1937
Relative Quantification
2
1
0
***
***
control
DMSO
0.5M M344
1.0M M344
5.0M M344
2 μg/ml cisplatin
cisplatin + 0.5M M344
cisplatin + 1.0M M344
cisplatin + 5.0M M344


A2780s
Relative Quantification
2
1
0
***
control
DMSO
0.5M M344
1.0M M344
5.0M M344
2 μg/ml cisplatin
cisplatin + 0.5M M344
cisplatin + 1.0M M344
cisplatin + 5.0M M344


A2780cp
Relative Quantification
2
1
0
**
**
control
DMSO
0.5M M344
1.0M M344
5.0M M344
2 μg/ml cisplatin
cisplatin + 0.5M M344
cisplatin + 1.0M M344
cisplatin + 5.0M M344

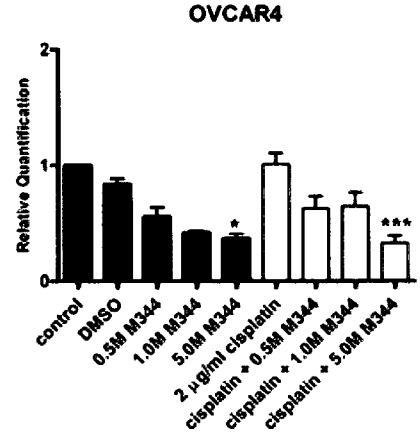

OVCAR4
Relative Quantification
2
1
0
*
***
control
DMSO
0.5M M344
1.0M M344
5.0M M344
2 μg/ml cisplatin
cisplatin + 0.5M M344
cisplatin + 1.0M M344
cisplatin + 5.0M M344

Figure 12: Treatment with the HDAC inhibitor M344 reduces BRCA1 mRNA expression in breast and ovarian cancer cell lines. BRCA1 mRNA levels in a panel of cell lines analyzed by real time quantitative RT-PCR following 24 hours of treatment with 0.5, 1.0, or 5.0 μM M344 either alone or in combination with 2 μg/ml cisplatin. Numbers represent the mean +/- SEM of three separate experiments. Differences between treatments were analyzed using 1-way ANOVA with Bonferroni posttest. Statistically significant differences, when present, between 5 μM M344 and DMSO control or 2 μg/ml cisplatin + 5 μM M344 and 2 μg/ml cisplatin alone are indicated by * where $P<0.05$, ** where $P<0.01$, and *** where $P<0.001$.

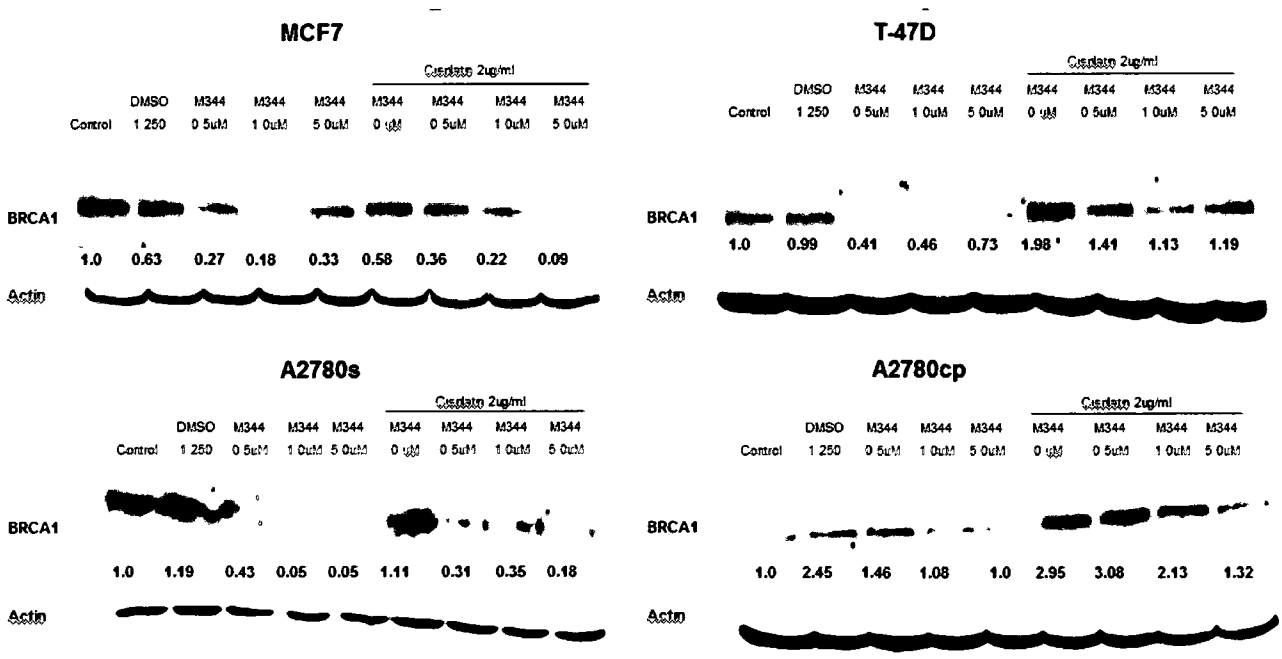

MCF7
Cisplatin 2ug/ml
DMSO M344 M344 M344 M344 M344 M344 M344
Control 1 25D 0 5uM 1 0uM 5 0uM 0 uM 0 5uM 1 0uM 5 0uM
BRCA1
1.0 0.63 0.27 0.18 0.33 0.58 0.36 0.22 0.09
Actin
T-47D
Cisplatin 2ug/ml
DMSO M344 M344 M344 M344 M344 M344 M344
Control 1 25D 0 5uM 1 0uM 5 0uM 0 uM 0 5uM 1 0uM 5 0uM
BRCA1
1.0 0.99 0.41 0.46 0.73 1.98 1.41 1.13 1.19
Actin
A2780s
Cisplatin 2ug/ml
DMSO M344 M344 M344 M344 M344 M344 M344
Control 1 25D 0 5uM 1 0uM 5 0uM 0 uM 0 5uM 1 0uM 5 0uM
BRCA1
1.0 1.19 0.43 0.05 0.05 1.11 0.31 0.35 0.18
Actin
A2780cp
Cisplatin 2ug/ml
DMSO M344 M344 M344 M344 M344 M344 M344
Control 1 25D 0 5uM 1 0uM 5 0uM 0 uM 0 5uM 1 0uM 5 0uM
BRCA1
1.0 2.45 1.46 1.08 1.0 2.95 3.08 2.13 1.32
Actin

Figure 13: Treatment with the HDAC inhibitor M344 reduces BRCA1 protein in breast and ovarian cancer cell lines. Western blot analysis of BRCA1 in a panel of cell lines following 24 hours of treatment with 0.5, 1.0, or 5.0 μM M344 either alone or in combination with 2 μg/ml cisplatin. Numbers represent densitometry readings with untreated control used as the calibrator and set to a value of 1.0.

When BRCA1 mRNA levels were examined in the ovarian cancer cell lines, it was found that treatment with M344 alone decreased expression in all of the cell lines as compared to DMSO control, with the most significant decreases observed in the A2780s (Figure 12). Combination treatment of cells with cisplatin and M344 resulted in a significant decrease in BRCA1 mRNA levels with the highest concentration of M344, as compared to treatment with cisplatin alone, in all of the cell lines with the exception of A2780s, where no decrease was observed (Figure 12). Treatment with cisplatin resulted in an increase in BRCA1 protein levels, reflective of the engagement of the DNA damage response (Figure 13) (109, 110). Protein levels of BRCA1 were assessed only in the A2780s and A2780cp cells, as protein was not detectable by western blotting in the OVCAR4 cells (see Section 3.3). Contrary to the mRNA results, BRCA1 protein levels were decreased after treatment with M344 either alone or in combination with cisplatin in the A2780s cells (Figure 13). This was also observed in the A2780cp cells (Figure 13). Therefore, taken together, these results demonstrate that BRCA1 represents a novel target of HDAC inhibitors through inhibition of its expression. Combining agents that induce DNA damage, like cisplatin, with HDAC inhibitors may represent a novel therapeutic approach.

3.7 Treatment with the HDAC inhibitor M344 causes decreased binding of acetylated Histone 4 to the BRCA proximal promoter region.

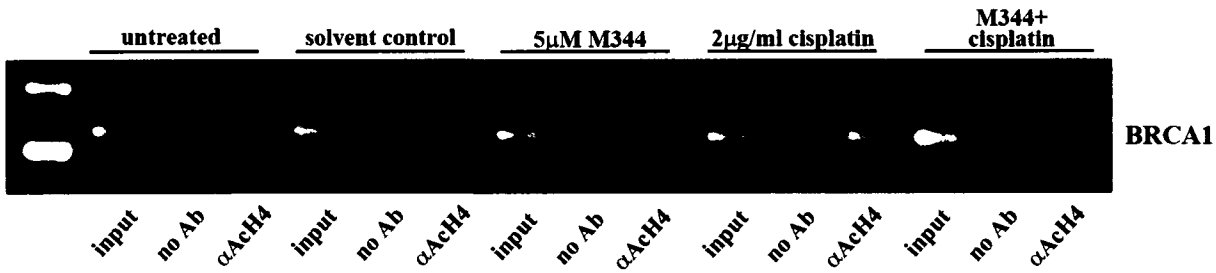
The mechanism by which the HDAC inhibitor M344 can down-regulate BRCA1 expression was investigated through the use of the chromatin immunoprecipitation (ChIP) assay. This assay was employed to determine whether the decrease in BRCA1 mRNA expression by HDAC inhibition was controlled at the transcriptional level.

Cells from the MCF7 breast carcinoma cell line were treated for 24 hours with M344 and cisplatin individually and in combination. MCF7 showed decreased levels of *BRCA1* proximal promoter DNA bound to acetyl-H4 (AcH4) following treatment with M344 alone relative to the control sample (Figure 14). The level of DNA bound to AcH4 increased in the samples treated with cisplatin alone (Figure 14). Importantly, *BRCA1* proximal promoter DNA levels bound to AcH4 decreased to levels below that seen in the single agent M344 treatment, in response to treatment with M344 and cisplatin in combination (Figure 14). These results indicate that the down-regulation of BRCA1 expression in response to M344 alone or in combination with cisplatin may occur at the transcriptional level. This experiment was also performed in the ovarian carcinoma cell line A2780s, with similar results.

3.8 The clinically approved HDAC inhibitor SAHA decreases BRCA1 protein expression *in vitro*.

The HDAC inhibitor M344 is effective in down-regulating BRCA1 expression and also increasing platinum-induced cytotoxicity. It is however, not approved for use in the clinic. Recently, the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, also known as vorinostat) has been approved for the treatment of cutaneous T-cell lymphoma (99). The down-regulation of BRCA1 expression, if any, following 24 hour SAHA treatment was analysed by western blot in A2780s cells. At all treatment concentrations, SAHA decreased BRCA1 protein levels as compared to the untreated controls (Figure 15F). Treatment with 1.0 μM SAHA was less effective in decreasing BRCA1 protein expression as compared with the same concentration of M344 (Figure 15F). At 5.0 μM, SAHA treatment decreased BRCA1 protein expression to

A


untreated
solvent control
5μM M344
2μg/ml cisplatin
M344+ cisplatin
BRCA1
input
no Ab
αAcH4

B

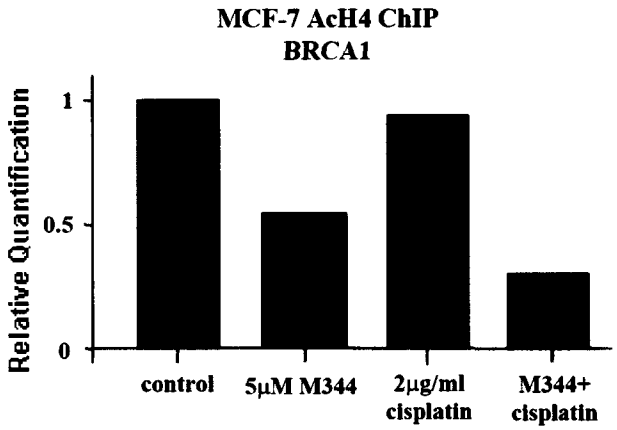
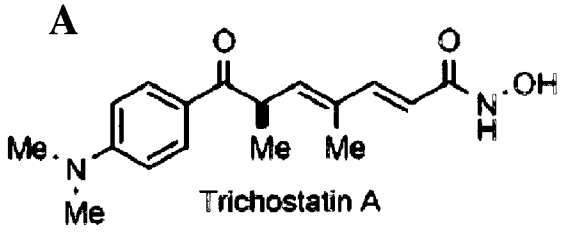

MCF-7 AcH4 ChIP
BRCA1
Relative Quantification
1
0.5
0
control
5μM M344
2μg/ml cisplatin
M344+ cisplatin

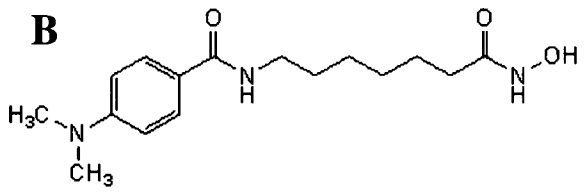
Figure 14: Treatment with the HDAC inhibitor M344 is associated with decreased acetylated Histone 4 at the *BRCA1* proximal promoter region. A. Real-time PCR products were run on a 1.6% agarose gel. Three samples of each treatment are seen: the input controls which have not been exposed to either agarose beads or AcH4 antibody, the Beads control samples to which agarose beads have been added in the absence of AcH4 antibody, the AcH4 to which agarose beads and AcH4 has been added. This final sample is the ChIP sample. B. MCF7 cells treated with an HDAC inhibitor alone or in combination with cisplatin (CDDP) show reduced amounts of *BRCA1* promoter DNA bound to acetylated histone 4 as quantified by real-time PCR suggesting a decrease in transcription of the *BRCA1* gene.

A



Trichostatin A

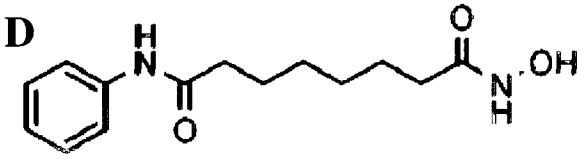
B



M344

C

D



Suberoylanilide hydroxamate (SAHA)

E

F

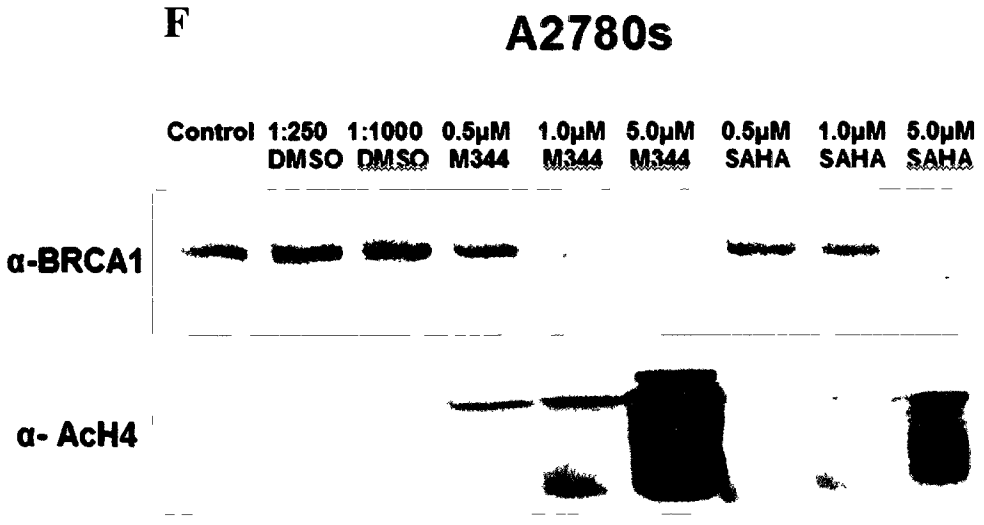


Figure 15: The clinically approved HDAC inhibitor SAHA decreases BRCA1 protein expression *in vitro*. A comparison of the chemical structures of the HDAC inhibitor TSA (A), HDAC inhibitor used for *in vitro* experiments in this study, M344 (B), and the HDAC inhibitor approved for clinical use, SAHA (D). C. The basic chemical structure shared by all three of these compounds consists of an enzyme binding region, a spacer region, and an enzyme inhibiting group. (Adapted and manipulated from Remiszewski, S. W., Sambucetti, L. C., Atadja, P., Bair, K. W., Cornell, W. D., Green, M. A., Howell, K. L., Jung, M., Kwon, P., Trogani, N., and Walker, H.Inhibitors of Human Histone Deacetylase: Synthesis and enzyme and cellular activity of straight chain hydroxamates. J Med Chem, *45*:753-757, 2002. and Jung, M., Brosch, G., Kolle, D., Scherf, H., Gerhauser, C., and Loidl, P. Amide analogues of trichostatin A as inhibitors of histone deacetylase and inducers of terminal cell differentiation. J Med Chem, *42*:4669-4679, 1999.) E. The hyperacetylation of histone 4 in Friend leukemic cells following treatment with the indicated HDAC inhibitors. (Adapted and manipulated from Jung, M., Brosch, G., Kolle, D., Scherf, H., Gerhauser, C., and Loidl, P. Amide analogues of trichostatin A as inhibitors of histone deacetylase and inducers of terminal cell differentiation. J Med Chem, *42*:4669-4679, 1999.) F. Levels of BRCA1 and acetyl-H4 (AcH4) protein as analyzed by Western blot following 24 hour treatments as indicated in A2780s cells.

undetectable levels which appears to be more effective than the same concentration of M344 (Figure15F). Therefore, the clinically approved HDAC inhibitor SAHA appears to down-regulate BRCA1 expression *in vitro*.

4. DISCUSSION

Ovarian cancer is the leading cause of death from gynaecologic malignancies (111). Poor prognosis is generally due to the development of chemoresistance to standard platinum-based therapies (112). Discovering chemotherapeutics that enhance response to platinum therapy is essential to aid in improving the 5-year survival rate of this disease, a dismal 30-40% that has not changed in decades (1). The purpose of this study was to enhance platinum-induced cytotoxicity in EOC by targeting BRCA1 using novel therapeutic approaches. Studies have shown that ovarian cancer patients with *BRCA1* germline mutations demonstrate improved response to platinum-based chemotherapy and improved overall survival as compared to those without the mutation (113). As BRCA1 is a critical component of the DNA-damage recognition and repair machinery, the inability of deficient tumour cells to repair the damage induced by platinum treatment results in increased apoptosis, translating into improved response. Results presented here demonstrate that the addition of an inhibitor of HDAC activity, M344, potentiates cisplatin treatment in a panel of breast and ovarian cancer cell lines via the targeting of the *BRCA1* tumour suppressor gene.

The first objective of this study was to identify a small molecule inhibitor that sensitizes EOC cells to platinum-induced cytotoxicity. None of the three small molecule inhibitors tested showed any activity when combined with the control chemotherapeutic agent, paclitaxel. Neither the inhibitor of the PARP1 enzyme nor the Cdk2 kinase showed any activity in combination with either platinum agent, as shown by the cell viability assay. In an IHC study of PARP1 expression in a cohort of ovarian serous carcinomas, strong PARP1 expression was demonstrated in 76% of

cases and this higher level of expression correlated with a poorer outcome compared to patients with comparatively low expression (68). The DNA lesions created by the inhibition of PARP1 require functional BRCA1 for recognition and repair (114). Therefore, if BRCA1 is present in cells, the lesions will be repaired. PARP1 is a rational therapeutic target in BRCA1-deficient cancer cells since BRCA1 dysfunction sensitizes cells to PARP1 inhibition by causing the accumulation of DSBs resulting in cell cycle arrest in G_2 phase, followed by apoptosis (68, 114, 115). In the present study, both A2780s and A2780cp cell lines exhibited relatively high levels of BRCA1 expression. Therefore, it is not unexpected that treatment with a PARP inhibitor did not demonstrate *in vitro* cytotoxicity. Although, an immunofluorescence assay visualizing DSBs by probing for phosphorylated H2AX would be a positive control experiment to confirm the function of the PARP inhibitor. Similarly, the inhibition of CDKs has shown increased levels of toxicity in cells deficient in components of DNA repair such as BRCA1 and ATM. Cells deficient in genes like BRCA1, commonly present in familial breast cancer cases, are up to 4-fold more sensitive to CDK inhibition (72). Cancers with mutated BRCA1 have been shown to select for activation of Cdk2 activity through the loss of the Cdk2-inhibiting p27 (116, 117). It has been suggested that Cdk2 inhibitors be specifically used in the clinic on patients with BRCA1-deficient tumours (72). Therefore, the inactivity of the Cdk2 inhibitor when in combination with the platinum agents in the BRCA1-expressing A2780s and A2780cp cell lines is not unexpected. In addition, analysis by flow cytometry could determine whether the Cdk2 inhibitor is functional by visualizing the cell cycle profile of the cells following treatment.

DNA repair pathways are frequently defective in malignant cells and targeting such defects seems to be a more rational therapeutic approach than direct gene therapy (118, 119). HDAC inhibitors are a promising new class of anticancer therapeutics that have been tested in phase I and phase II clinical trials (97-99). The interaction between HATs and HDAC enzymes regulates gene expression by modifying chromatin transcription in various malignancies, and BRCA1 is known to play a role in altering chromatin structure (38). HDAC inhibitors have shown some promise as single agent therapies for EOC, though they may be most useful as an addition to standard therapies. There is also evidence that HDAC inhibitors could function in part via targeting BRCA1 expression through BRCA1 involvement in chromatin remodelling (38). The cell viability assay used in this study showed a trend towards increased cytotoxicity with the addition of an HDAC inhibitor to cisplatin and carboplatin in the paired A2780s and A2780cp EOC cell lines. The mechanism by which HDAC inhibition is thought to enhance platinum-induced cytotoxicity is through chromatin relaxation, allowing for increased DNA damage in transcriptionally active DNA regions (91). This study demonstrates that in addition to this mechanism, the HDAC inhibitor M344 may sensitize the A2780s and A2780cp cells to platinum by decreasing the mRNA and protein expression of BRCA1, as shown by quantitative RT-PCR and Western blot analysis. This represents a novel mechanism of action of this class of agents whereby the HDAC inhibitors can target expression of the DNA repair protein, BRCA1 in combination with platinum agents. This phenomenon requires further investigation to determine its scope in a variety of

human cancers and to delineate its mechanism with respect to targeting BRCA1 expression.

In order to expand the observations of the HDAC inhibitor activity in combination with platinum agents, a panel of three breast carcinoma (T47-D, MCF7, and HCC1937) and three ovarian carcinoma cell lines (A2780s, A2780cp, and OVCAR4) were included in this study. Variable levels of BRCA1 mRNA and protein were observed in this ovarian and breast cancer cell line panel when analyzed by quantitative RT-PCR and western blot analysis. This is consistent with the range of expression levels our group and others have observed in ovarian and breast tumour specimens (4, 13, 28, 120). HCC1937 cells harbour the germline BRCA1 mutation 5382insC, which causes in a frameshift creating a premature stop codon and resulting in a truncated non-functional protein product (108). As anticipated, the HCC1937 breast carcinoma cells did not express any BRCA1 protein. HCC1937 cells did have detectable levels of BRCA1 mRNA, though it was lower than the other breast cancer cell lines examined, which is in keeping with previous observations that tumours from germline mutation carriers express mRNA levels significantly lower than those seen in sporadic tumours (121). Of all cell lines examined, OVCAR4 exhibited the least BRCA1 mRNA when analyzed. The protein amount was also comparatively low. The aforementioned variable levels of basal BRCA1 mRNA and protein expression would be useful to assess the role, if any, of this gene in the activity of the HDAC inhibitors when in combination with platinum agents.

Studies conducted both *in vitro* and *in vivo* have demonstrated some cytotoxic efficacy of HDAC inhibitors in ovarian cancer models (93, 104, 122-126) A recent

clinical study however, have shown minimal value of these compounds as a single agent treatment in this disease (127). HDAC inhibitors have also been shown, in ovarian and other cell types, to enhance the effect of radiotherapy and some types of chemotherapy (103, 128, 129); however, the molecular mechanism behind this effect is not well understood. The work presented here has shown that co-treatment of the ovarian cancer cell lines A2780s and A2780cp with the HDAC inhibitor M344 increased sensitivity to cisplatin. Upon further investigation, this study confirmed that the cisplatin + M344 co-treatment of a panel of ovarian and breast cancer cell lines was more effective than treatment with either agent alone in the cells. Both the breast (T-47D) and the ovarian (A2780s) lines with higher BRCA1 levels displayed the greatest increase of cisplatin cytotoxicity with the addition of M344. No effect was observed in the BRCA1-deficient HCC1937 cells and little to no effect in the low-expressing OVCAR4 cells. Flow cytometry confirmed the increased apoptosis seen in the cisplatin + M344 combination treatment with the cell viability assay. Considering the panel of cell lines, cells with low levels of BRCA1 were, in general, more sensitive to single agent cisplatin treatment than those with higher levels, consistent with the clinical observation that BRCA1 levels in tumours are predictive of chemoresponse in ovarian cancer (13, 60). Thus, co-treatment with an HDAC inhibitor was able to potentiate the effect of cisplatin in breast and ovarian cancer cells that displayed minimal response to cisplatin treatment alone, which could be a result of targeting of BRCA1 by M344.

The BRCA1 tumour suppressor gene plays a major role in the detection and repair of DNA damage (39, 40, 130). Without functional BRCA1 activity, cells demonstrate

an increase in irreparable DNA double-strand breaks in response to DNA-damaging agents, such as chemotherapeutics, due to a shift from the HR pathway to the more error prone NHEJ pathway (4). In order to determine if treatment with M344 was affecting BRCA1 mRNA and protein levels, the panel of ovarian and breast cancer cell lines was treated with M344 alone or in combination with cisplatin and expression levels were assessed via quantitative RT-PCR and western blotting. BRCA1 protein levels were found to be decreased by treatment with M344 alone in the entire cohort of cell lines examined. The effect was more pronounced in cells with higher BRCA1 protein levels. Treatment with cisplatin caused an increase in BRCA1 protein expression, reflective of the engagement of the DNA damage response triggered by the DSBs caused by this treatment (109, 110). Treatment with M344 in conjunction with cisplatin reduced BRCA1 protein levels in all cell lines assessed, thus counteracting the effect of cisplatin treatment alone. In the ovarian cancer cell lines, a significant decrease was observed in 2 of the cell lines OVCAR4 and A2780cp, with the exception being the A2780s cells. A2780s cells have wildtype p53 status and are exquisitely sensitive to cisplatin treatment, whereas A2780cp are mutant for p53 and minimally sensitive to cisplatin. OVCAR4 cells are quite sensitive to cisplatin treatment, but are also mutant for p53 (131). Thus, in ovarian cancer cells, p53 status may be an important determinant of the ability of an HDAC inhibitor to target BRCA1 in the presence of DNA-damaging agents, and is a phenomenon that warrants further investigation. The targeting of BRCA1 expression by HDAC inhibitors was first reported by Zhang *et al*, who observed that the HDAC inhibitor

TSA sensitized resistant human squamous carcinoma cells to radiation-induced DNA damage and apoptosis (102).

It is possible that the decrease in BRCA1 mRNA expression as seen in the quantitative RT-PCR experiments could be due to decreased mRNA stability following treatment with the HDAC inhibitor. To eliminate this possibility and determine whether this effect is due to transcriptional regulation as controlled by the accumulation of acetylated histones, a ChIP assay was performed on cells treated with each agent alone and in combination. As previously mentioned, genes actively being transcribed are known to be associated with nucleosomes with high levels of acetylated histones and silent genes are associated with nucleosomes with low levels of acetylated histones (87-89). It would be expected then, that the results seen earlier with the decrease in BRCA1 mRNA and protein expression following HDAC inhibitor treatment alone and in combination with cisplatin would be due to the BRCA1 promoter DNA being associated with fewer acetylated histones. The results of the ChIP assay confirm this hypothesis and mirror the pattern of BRCA1 mRNA and protein expression following these treatments. The amount of BRCA1 promoter DNA bound to acetylated histone decreased in the M344 single agent treatment relative to controls, which indicates that transcriptional repression may be occurring. The amount of BRCA1 DNA bound to acetylated histone increased following cisplatin treatment, indicating that the increase in BRCA1 mRNA and protein expression is due to transcriptional up-regulation as caused by being associated with acetylated histones. These results are in keeping with the previous observation that an increase in BRCA1 expression is reflective of the activation of the DNA damage

response triggered by the platinum agents (109, 110). In the HDAC inhibitor – cisplatin treated cells, the amount of BRCA1 DNA bound to acetylated histone was decreased. Therefore, the ChIP assay supports that the decrease in BRCA1 expression following HDAC inhibitor treatment, either alone or in combination, may be transcriptionally regulated.

HDAC inhibitor treatment could be affecting the regulation of BRCA1 transcription by the role of acetylated histones in modulating chromatin structure. HDAC inhibitors promote the accumulation of acetylated histones within the nucleosomes. This leads to the loosening of the chromatin structure and actively transcribed genes because the loosened structure allows access for the transcriptional machinery (84, 87-89). The ChIP assay results indicate that by decreasing BRCA1 promoter DNA bound to acetylated histones, this section of chromatin is more condensed and therefore, less transcriptionally active. The primers used in this particular study amplify a small stretch of the BRCA1 proximal promoter region for DNA fragments approximately 1 kb in size and while the PCR fragment does not encompass the negative transcriptional element contained within this region (the UP site), the entire BRCA1 promoter would be analyzed by this approach (Figure 3) (52, 106). HDAC inhibitor treatment may allow for the expression of an as yet, undetermined repressor protein which targets the BRCA1 gene. Recently, metastasis-associated tumour antigen 1 (MTA1) has been identified as a transcriptional repressor of BRCA1 (132). Molli *et al* showed that MTA1, as a component of the nucleosome remodelling and deacetylating (NuRD) complex, targeted the ERE contained within exon 1 of the BRCA1 gene, which implicates MTA1 as a novel transcriptional

repressor of BRCA1 (132). However, their results show that following treatment with the HDAC inhibitor TSA, MTA1-mediated BRCA1 repression was relieved, which indicates that MTA1 may not be the mechanism by which HDAC inhibitors are targeting BRCA1 as observed in the work published by our group as well as others (13, 102, 132). Hence, it is most likely that the primary effect of the HDAC inhibitor treatment combination in the cell lines analysed in this study is the condensation of a section of chromatin containing the proximal promoter region, resulting in repression of BRCA1 expression through an as yet unknown mechanism.

The results which have been shown here present a possible alternative to the current platinum- and taxane-based therapeutic regimen in the clinic. However, the HDAC inhibitor which has shown successful and safe activity in the clinic is a structural analogue of TSA and M344, SAHA (Figure 15A – C) (112, 122, 133-135). SAHA is approved for the treatment of cutaneous T-cell lymphoma (112). It was imperative to confirm activity of SAHA in a model of EOC and results shown here indicate that the targeting of BRCA1 expression may be a common mechanism of action of HDAC inhibitors, as supported by the work presented by Zhang *et al* with TSA (102). It was expected that SAHA would have slightly weaker activity *in vitro* due to toxicological and chemical data regarding TSA, M344, and SAHA (133-135). The observed inhibitory activity of SAHA is thirty-fold weaker than that of TSA (136). In comparison, a thirty-fold greater concentration of M344 is required to achieve histone acetylation levels comparable to that of TSA (Figure 15E) (134). The HDAC inhibitor M344 is structurally related to both TSA and SAHA, where M344 possesses an enzyme binding region similar to TSA and a spacer region as well as

enzyme inhibiting group similar to SAHA (Figure 15A – D) (134, 135). There are currently clinical trials recruiting ovarian cancer patients to assess combinatorial treatment with carboplatin and SAHA. It would be of great interest to observe whether tumour BRCA1 levels influence outcome in this treatment setting.

In the future, there are several avenues available to expand on the observations made in this study. An experiment in which a wild-type BRCA1 is ectopically expressed in the BRCA1- deficient HCC1937 cell line would determine if the potentiation of platinum-induced cytotoxicity by the HDAC inhibitors is due to its ability to target BRCA1. Zhang *et al* observed that while TSA had no effect on the radiosensitivity of the BRCA1-null HCC1937 cell line, TSA treatment increased the radiosensitivity of HCC1937 cells ectopically expressing wild-type BRCA1, which implies that the TSA-induced sensitization effect is due to its ability to target BRCA1 (102). The opposite experiment could also prove useful. The effect of silencing this gene through BRCA1-targeted RNA interference on the HDAC inhibitor-induced potentiation of platinum sensitivity in a BRCA1 expressing cell line could be used to confirm whether the activity of the HDAC inhibitor is dependent on its targeting BRCA1. A cell line such as A2780s or MCF7 could be used for this purpose. In addition, the regulation of BRCA1 transcription by HDAC inhibition remains to be deciphered. Whether the HDAC inhibitor treatment causes the down-regulation of BRCA1 expression by chromatin remodelling or indirectly resulting in its down-regulation by providing access of BRCA1-targeting transcriptional repressors to the gene would be worthy of investigation. Identification of this mechanism could not only lead to the development of BRCA1-specific targeted therapies and have far-

reaching implications in the treatment of BRCA1-related tumours, but also reveal the mechanisms dictating the expression of this crucial DNA repair gene. In addition, the HDAC inhibitors discussed here (TSA, SAHA, and M344) are broad spectrum inhibitors, which means they target HDAC enzymes of all four classes (94). It is known that BRCA1 interacts with HDAC1 and HDAC2 which are HDAC isoforms of Class I which in turn, have been shown to be overexpressed in ovarian tumour tissues (93, 94). The use of a Class I-specific inhibitor such as MS-275 or even the HDAC1/HDAC2-specific inhibitor depsipeptide in combination with the platinum agents would be of great importance in determining the role of HDACs and perhaps, their interaction with BRCA1 in EOC. In hindsight, the analysis of HDAC expression levels in the group of cell lines in this study, in addition to that of BRCA1, was imperative to interpreting the effects of the HDAC inhibitors in combination with the platinum agents.

The overall objective of this study was to uncover novel therapeutic avenues to overcome chemoresistance in ovarian cancer. In summary, HDAC inhibitors show promise as therapeutic agents in combination with platinum-based drugs in the treatment of breast and ovarian cancers due to their ability to down-regulate BRCA1 expression. This study has shown that the inhibition of HDACs sensitizes ovarian cancer cells to platinum chemotherapeutics but not taxane chemotherapeutics. Inhibition of Cdk2 or PARP does not sensitize cells to either family of chemotherapeutics. A novel finding of this study suggested that HDAC inhibition effectively targeted mRNA and protein expression of BRCA1 *in vitro,* both alone and in combination with platinum agents. This study also demonstrates that in breast and

ovarian cancer cell lines, HDAC inhibition showed a trend towards increasing platinum-induced cytotoxicity. HDAC inhibitor treatment appeared to cause decreased binding of acetylated histone to the BRCA1 proximal promoter region, which may be leading to the down-regulation of BRCA1 expression. *In vitro* experiments presented here show that the clinically relevant HDAC inhibitor, SAHA, is effective in targeting BRCA1 protein expression. This is significant as lower BRCA1 levels have been shown to be a positive predictive marker of progression-free survival and overall survival in ovarian cancer (13). Thus, co-treatment with an HDAC inhibitor and a platinum-based agent may provide an effective novel treatment for ovarian cancer via the targeting of BRCA1.

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APPENDICES

Histone deacetylase inhibition targets BRCA1 expression: Potential novel therapeutic implications

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Abbreviations: OC: ovarian cancer, HDACi: histone deacetylase inhibitor, BRCA1: breast cancer 1,

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Abstract

The improved outcome of Breast-Cancer 1 (BRCA1)-deficient breast and ovarian cancer is linked to their impaired ability to repair DNA damage Targeting BRCA1 expression can sensitize tumour cells to platinum-based chemotherapies However, therapeutically relevant agents that target BRCA1 expression have not been identified Here we explore the effect of histone deacetylase inhibition (HDACi) on BRCA1 expression and platinum sensitivity in a panel of breast and ovarian cancer cell lines BRCA1 mRNA and protein expression was determined by Q-PCR and Western blot The efficacy of HDACi to potentiate the cytotoxicity of platinum-based chemotherapeutics was evaluated using the MTT viability assay and flow cytometry The effect on DNA damage was measured by immunofluorescence staining and flow cytometry for γH2A X foci Baseline BRCA1 expression was variable in three breast (MCF7, T47D and HCC1937) and three ovarian (A2780s, A2780cp and OVCAR4) cancer cell lines Expression of BRCA1 protein decreased in response to the HDACi M344 in the cell lines that expressed detectable levels of BRCA1 by Western blot BRCA1 mRNA levels decreased with the addition of M344 in all cell lines evaluated Treatment with M344 increased the sensitivity to cisplatin and carboplatin treatment in the cell lines with higher BRCA1 levels Expression of BRCA1 protein and mRNA was decreased in cells treated with the HDACi and cisplatin A2780s cells subjected to combination platinum and HDACi treatment demonstrated increased levels of DNA damage This study supports a novel mechanism of BRCA1 targeting with the potential to sensitize breast and ovarian cancer cells to platinum treatment

Introduction

Chemoresistance is primary obstacle in the management of advanced epithelial ovarian cancer (OC) and novel therapies are required to enhance standard platinum-based treatment and to improve the poor prognosis In various *in vitro* and retrospective clinical studies, it has been suggested that the Breast-Cancer 1 (BRCA1) protein is a marker of chemoresistance and that BRCA1-deficient tumors predict for a better overall survival after standard platinum-based chemotherapy (1-3) The improved outcome in BRCA1-deficient tumors is believed to be due in part, to an increased sensitivity to DNA damaging chemotherapeutics, such as cisplatin, as a result of the induction of irreparable DNA double strand breaks (DSB) through the homologous recombination repair (HR) pathway Thus, BRCA1 has been regarded as a rational therapeutic target to help overcome platinum resistance in advanced and recurrent OC While phase I and phase II clinical trials of direct gene therapy to inhibit BRCA1 expression were not promising (4, 5), in an era of evolving molecular inhibitors, new therapeutic strategies merit consideration

BRCA1 is involved in a number of cellular processes including the repair of DNA damage, usually through the error free mechanism of HR (6, 7) BRCA1 functions as a scaffolding protein, colocalizing with complexes of other DNA damage repair proteins at sites of damage (8) Cells deficient in BRCA1 resort to more error-prone mechanisms of repair such as non-homologous end-joining (NHEJ) which predisposes to genomic instability and renders cells susceptible to cytotoxic stress (9) Ovarian cancer patients with germline BRCA1 mutations display an improved response to DNA-damaging

platinum based therapy (10, 11), as do Brca1-deficient mouse ovarian surface epithelial cells (12).

The interaction between histone acetyl transferase (HAT) and histone deacetylase (HDACs) enzymes modulates chromatin structure and regulates transcription factor accessibility to regulate gene expression (13). Chromatin remodeling is particularly active during the DNA damage response, with rapid, localized unwinding of chromatin to allow for the entry and assembly of proteins that form DNA repair complexes (14). During this damage-induced remodeling, HAT complexes work with ATP-dependent chromatin remodeling complexes to facilitate repair. Chromatin structure also influences the susceptibility of a DNA sequence to damage, with areas of loosely compacted, and hence more transcriptionally active chromatin increasing the frequency of DSBs (14).

The BRCA1 tumour suppressor gene also plays a role in chromatin remodeling. The BRCT domain of BRCA1 gene associates with both HDAC1 and HDAC2 and it is thought that this association directly represses transcription (15). BRCA1 has been shown to be a component of the SWI/SNF chromatin remodeling complex, and its coactivation function with p53 is mediated through this complex (16). It is thought that the BRCA1-SWI/SNF complex may be essential for the activation of genes involved in the DNA damage response and that it could also play a more direct role in homologous repair by enabling access to sites of damage via chromatin remodeling (16).

HDAC inhibitors (HDACi) promote the accumulation of acetylated histones, which results in a relaxed chromatin structure (17), potentially allowing better access to DNA damaging agents. They have shown promise as anticancer agents, demonstrating inhibition of growth of cancer cells both *in vitro* and *in vivo* (18, 19), and recently the

HDACi suberoylanilide hydroxamic acid (SAHA, also known as vorinostat) has been approved for the treatment of cutaneous T-cell lymphoma (20). Because of their ability to relax chromatin structure and hence render DNA more vulnerable to potential assaults, HDACi are being evaluated as combination therapies in conjunction with standard cytotoxic treatments. A number of HDAC inhibitors are reported to increase radiation sensitivity in various tumor types (21-23) and it is suggested that HDAC inhibitors work in part by inhibiting DNA repair. Evidence exists that HDAC enzymes are important for homologous recombination repair of DNA double-strand breaks and the assembly of RAD51 subnuclear foci (24). The BRCA1-deficient breast cancer cells, HCC1937 demonstrated increased sensitivity to IR (25). In the same cell line, Zhang et al have shown that trichostatin A exposure delayed DNA damage repair in response to IR by the suppression of key genes including BRCA1 (23). HDACi have also demonstrated the ability to potentiate the effects of numerous chemotherapeutic agents in a pre-clinical setting, such as topoisomerase inhibitors, and DNA-damaging agents such as platinum compounds (17).

HDACi have shown potential as a therapeutic option in ovarian cancer and their ability to target BRCA1 makes them an even more attractive option for this disease (26-28). Our group has recently demonstrated that HDACi also sensitize A2780 OC cells to platinum by decreasing the expression of BRCA1 (3). In this study, we observe the effect of the HDACi M344 on the cytotoxicity of various breast and ovarian cancer cell lines in combination with platinum compounds.

Materials and Methods

Cell Culture. The A2780s and A2780cp cell lines were kindly provided by Dr. B. Vanderhyden (Ottawa Hospital Research Institute, Ottawa, ON, Canada) and the T47D, BT-549, and OVCAR-4 cell lines were donated by Dr. J. Bell (Ottawa Hospital Research Institute, Ottawa, ON, Canada). MCF7, HCC-1937 and SKOV-3 were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in Dulbecco's-MEM (Media Services, Ottawa Regional Cancer Centre, ON, Canada) supplemented with 10% fetal bovine serum (Wisent Inc., St-Bruno, Quebec, Canada) and 100ug/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA). Unless otherwise described, cells were treated for 24 hours with 2 μg/ml cisplatin (provided by the pharmacy at the Ottawa Hospital Regional Cancer Centre, Ottawa, Ontario, Canada) alone and in combination with the HDAC inhibitor M344 (Biovision, Mountain View, CA) at concentrations of 0.5, 1.0, or 5.0 μM .

RNA isolation and RT-PCR. Total RNA was extracted from cell lines in sub-confluent 10cm dishes using the RNeasy® kit (Qiagen, Germantown, MD). RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc, Wilmington, DE). Total RNA (1μg) was reverse-transcribed for polymerase chain reaction (PCR) as previously described. The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification. A real-time PCR reaction was carried out in a total volume of 25 μl that contained 2.5 μl of synthesized cDNA (42ng), 1.25 μl of TaqMan Gene Expression Assay Primer/Probe (20X) (Applied Biosystems, BRCA1, HS00173233), 12.5 μl of TaqMan Universal PCR Master Mix (2X) (Applied Biosystems) and 8.75 μl of RNase-free water for BRCA1

expression. GAPDH (Applied Biosystems, HS4333764-F) was used as an endogenous control. Amplification conditions were 95°C for 5 min, 40 PCR cycles at 95°C for 15 sec, and 60°C for 1 min. Three independent reactions from separate RNA extractions were used to determine the average RNA expression and a standard error for each treatment condition.

Western Blotting. Protein samples were collected in RIPA buffer containing 1X Protease Inhibitor Cocktail (Sigma-Aldrich, St-Louis, MO) and protein content was measured using a commercially available protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL) and a Biomate3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on 8-12% SDS polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA). Blocking was carried out with 5% milk in Tris-buffered saline with Tween-20 (TBS-T). For all subsequent immunoblotting, antibodies were diluted to the appropriate concentration in 5% milk in TBS-T. Blots were incubated with the following primary antibodies for 1 hour at room temperature or overnight at 4°C; mouse-anti BRCA1 (1:200, D-9, Santa Cruz, Santa Cruz, CA), rabbit anti-p53 (1:1000, FL-393, Santa Cruz), rabbit-anti acetylated Histone 4 (acetyl H4) (1:1000, Upstate Cell Signaling, Lake Placid, NY) and mouse-anti actin (1:5000, Sigma-Aldrich). Following three washes in TBS-T, blots were incubated with the appropriate HRP-labeled secondary antibody (goat-anti-rabbit-HRP, goat-anti-mouse-HRP, 1:5000, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. The chemiluminescent substrate used was Supersignal West Pico (Pierce) and the visualization of the protein bands was performed using the GeneSnap image

acquisition system followed by densitometry analysis with the GeneTools software (Syngene, Frederick, MD).

Cell Viability Assay. Cell viability was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) rapid colorimetric assay. Approximately 4,500 cells were seeded into each well of a 96-well flat bottom plate. The cells were incubated overnight to allow for cell attachment. Cells were then treated with cisplatin in concentrations of 0-8 μg/ml alone or in combination with 1 μM of the HDAC inhibitor M344. Forty eight hours following treatment, 42μl of a 5mg/ml solution in phosphate buffered saline of the MTT substrate (Sigma-Aldrich) was added and incubated for up to 4 hrs at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 82μl of a 0.01M HCl/10% SDS (Sigma-Aldrich) solution and plates were incubated overnight at 37°C. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies (Chantilly, VA) at 570 nm to determine the optical density of the samples.

Flow Cytometric Analysis of Apoptosis. Cells treated for 48 hours in 10cm dishes were fixed in 80% ethanol for 1 hour. Cells were then washed with PBS and resuspended in staining buffer (0.2% Triton X-100, 1 mM EDTA in PBS, pH7.4) containing 25μg/mL propidium iodide (Sigma-Aldrich) and 100 μg/mL RNaseA (Sigma-Aldrich). Cells were incubated with staining buffer in the dark for 1 hour prior to DNA quantification by the Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Data analysis was performed using ModFit *LT* (Verity Software House Inc., Topsham, ME).

Immunofluorescence. Cells were fixed on gelatin-coated coverslips in cold methanol at -20°C for one hour, followed by three washes in 1X PBS. The cells were then permeabilized via incubation with 0.2% Triton-X-100 in PBS for 10 min, followed by 3

washes in PBS. Blocking was carried out for 30 min at room temperature with 5% normal goat serum in PBS. Cells were incubated with mouse anti-H2A.X (ser139) (1:100 in PBS, Millipore) for one hour, followed by three PBS washes. Secondary antibody, anti-mouse Alexa Fluor 488, (Invitrogen, 1:400 in PBS) was applied for one hour, followed by 3 washes in PBS. Following a rinse with ddH2O, coverslips were mounted on glass slides using Vectashield mounting medium with DAPI (Vector Laboratories, Burlington, ON, Canada). Fluorescence was assessed using the Axioskop 2 MOT (Carl Zeiss MicroImaging, Thornwood, NY) microscope.

Flow Cytometric Analysis of γ-H2A.X Expression. Following treatment, cells were trypsinized, washed in PBS and fixed on ice with 1% paraformaldehyde for 15 min. Following centrifugation, the cell pellet was resuspended in 500 µl of PBS and transferred to a tube containing 4.5 ml of cold 70% ethanol and kept at -20°C for a minimum of 2 hours. Cells were centrifuged and then washed twice in BSA-T-PBS (1% bovine serum albumin and 0.2% Triton-X-100 in 1X PBS). Following the second wash, the cell pellet was resuspended in BSA-T-PBS containing mouse anti-H2A.X (ser139) (Millipore) primary antibody at 1:100 and incubated overnight at 4°C. Cells were then washed 1X in BSA-T-PBS and resuspended in BSA-T-PBS containing anti-mouse Alexa Fluor 488 (Invitrogen) secondary antibody at 1:400 and incubated at room temperature in the dark for one hour. Cells were washed 1X in BSA-T-PBS and resuspended in PBS containing 50 µl/ml propidium iodide (Sigma-Aldrich) and 5µl/ml RNAse A (Sigma-Aldrich). Cells were analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter) and the resulting data was assessed using ModFit software (Verity Software House).

Statistical Analysis. The probability of significant differences was determined by

analysis of variance (ANOVA; multiple groups). Bonferroni's posttest was used to determine significance between specific treatments when whole group differences where detected by ANOVA. For all analyses, significance was inferred at $P<0.05$ and P values were two-sided. Analyses were performed using Graphpad Prism statistical software (Graphpad Software, San Diego, CA).

Results

Expression of BRCA1 and p53 in a panel of breast and ovarian cancer cell lines.

Four breast cancer cell lines (MCF7, T-47D, BT549, and HCC1937) and four ovarian cancer cell lines (A2780s, A2780cp, SKOV3, and OVCAR4) were analyzed for BRCA1 protein expression via Western blot (**Figure 1A**) and mRNA expression by qPCR (**Figure 1B**). HCC1937 cells harbour the germline BRCA1 mutation 5382insC, which results in a frameshift causing a premature stop codon and resulting in a truncated non-functional protein product (29). The breast carcinoma line MCF7 was used as a positive control and was used as the calibrator for analysis of expression of both mRNA and protein levels and assigned an expression value of 1.0. Relative to itself, MCF7 cells displayed the highest levels of BRCA1 protein of the breast cancer cell lines. Predictably HCC1937 cells did not display any detectable BRCA1 protein. A2780s expressed the most protein of the ovarian cancer cell lines, but only slightly more than their cisplatin-resistant counterpart A2780cp cells. T-47D cells displayed the highest levels of BRCA1 mRNA expression of the breast lines, with almost twice the levels of the MCF7 cells. HCC1937 cells did demonstrate detectable levels of BRCA1 mRNA, comparable to those

of MCF7. A2780s cells expressed the most BRCA1 mRNA of the ovarian cells, with twice the expression level of its cisplatin-resistant pair.

Treatment with the HDAC inhibitor M344 enhances the efficacy of cisplatin treatment in breast and ovarian cancer cell lines. Effects on cell viability of treatment with M344 alone or in combination with cisplatin were measured using the MTT cell viability assay. Addition of M344 to cisplatin treatment did not enhance the effect of cisplatin in either the HCC1937 or the BT549 breast cancer cells (**Figure 2**). In MCF7 cells, cell viability was significantly reduced with the addition of M344 to cisplatin treatment, but only at the lowest dose of cisplatin used (1 μg/ml, $P < 0.05$) (**Figure 2**). Cell viability of the T-47D cells was significantly lower, by more than 50%, with the addition of M344 to cisplatin treatment at all the concentrations of cisplatin used ($P < 0.001$ at all concentrations, **Figure 2**). Treatment with M344 enhanced the effect of cisplatin treatment on cell viability in the A2780s cells at concentrations of 1 and 2 μg/ml cisplatin ($P < 0.05$). No effect was observed in the A2780cp cells (**Figure 2**). An enhancement of effect was only observed at a dose of 1 μg/ml cisplatin in the OVCAR4 ($P < 0.05$) and the SKOV3 ($P < 0.01$) ovarian cancer cells (**Figure 2**).

Treatment with the HDAC inhibitor M344 in combination with cisplatin increases apoptosis. Cells were treated with M344 and cisplatin, alone or in combination, for 48 hours and then subjected to flow cytometric analysis. Treatment with M344 alone did not cause a dramatic increase in apoptosis versus control cells. Treatment with cisplatin alone resulted in increased apoptosis in all cells examined as compared to controls. Treatment

of the cells with a combination of M344 and cisplatin resulted in an increase in apoptosis levels above what was observed with either agent alone, with the exception of MCF7 cells, where no such increase was observed (**Figure 3**). A2780cp cells demonstrated the highest levels of apoptosis in response to combination treatment with M344 and cisplatin. A2780s cells were too sensitive to cisplatin to be evaluated for apoptosis by flow cytometry after 48 hours of treatment and thus were not assessed.

Treatment with the HDAC inhibitor M344 reduces BRCA1 protein and mRNA expression in breast and ovarian cancer cell lines. mRNA levels of BRCA1 were assessed following exposure to M344 alone or in combination with cisplatin. In all of the breast cancer cell lines examined, treatment with M344 alone resulted in a significant decrease in BRCA1 mRNA expression at the highest concentration as compared to DMSO-treated controls (**Figure 4**). With the exception of MCF7, BRCA1 mRNA levels were increased with cisplatin treatment alone as compared to untreated control in all of the breast cancer cell lines. The addition of M344 to cisplatin treatment resulted in a decrease in BRCA1 mRNA levels in all of the breast cancer lines, though the result was not significant in the BT549 cells (**Figure 4**). BRCA1 protein levels were found to decrease with treatment with M344 alone as compared to DMSO control and were also found to decrease with treatment with cisplatin in combination with M344 as compared to cisplatin alone in all the breast cancer cell lines examined (**Figure 5**). Due to their mutant BRCA1 status, BRCA1 protein was not detectable in the HCC1937 cells and thus protein levels in response to treatment were not assessed in these cells.

When BRCA1 mRNA levels were examined in the ovarian cancer cell lines, it was found that treatment with M344 alone decreased expression in all of the cell lines as compared to DMSO control, with the most significant decreases observed in the A2780s and the SKOV3 cells (**Figure 4**). Combination treatment of cells with cisplatin and M344 resulted in a significant decrease in BRCA1 mRNA levels with the highest concentration of M344, as compared to treatment with cisplatin alone, in all of the cell lines with the exception of A2780s, where no decrease was observed (**Figure 4**) Protein levels of BRCA1 were assessed only in the A2780s and cp cells, as protein was barely detectable by western blotting in the SKOV3 and OVCAR4 cells Contrary to the mRNA results, BRCA1 protein levels were decreased with treatment with M344 either alone or in combination with cisplatin in the A2780s cells (**Figure 5**) This was also observed, to a lesser degree, in the A2780cp cells (**Figure 5**)

Treatment with the HDAC inhibitor M344 in combination with cisplatin results in an increase in DNA damage as assessed by γH2A.X foci formation. A2780s cells were treated with M344 alone or in combination with cisplatin and the impact of DNA damage levels, as assessed by γH2A X foci formation, were evaluated both by direct immunofluorescence and by flow cytometry Cells treated with DMSO as a control did not display any γH2A X foci Foci were observed in a small number of cells with treatment with 5 0M M344 The majority of cells displayed many foci when treated with cisplatin either alone or in combination with M344 (**Figure 6A**) Following treatment, cell samples were also sorted via flow cytometry after being incubated with a fluorescent-labeled anti γH2A X antibody (**Figure 6B**) Cells which were not treated with primary

antibody were used as a control. Minimal labeling of cells treated with M344 alone was observed. Cells treated with cisplatin alone displayed both labeled and unlabeled populations. The majority of cells treated in combination with both M344 and cisplatin were labeled for γH2A.X, indicating an increase in DNA damage.

Discussion

Ovarian cancer is the leading cause of death from gynecologic malignancies (30). Poor prognosis is generally the result of the development of chemoresistance to standard platinum-based therapies (31). The discovery of chemotherapeutics that enhance response to platinum therapy is essential to aid in improving the 5-year survival rate of this disease, a dismal 30-40% that has not changed in decades. Studies have shown that ovarian cancer patients with BRCA1 germline mutations demonstrate improved response to platinum-based chemotherapy and overall survival as compared those without this mutation (10). As BRCA1 is a critical component of the DNA-damage recognition and repair machinery, the inability of deficient tumour cells to repair the damage induced by platinum treatment results in increased apoptosis, translating into improved response. Direct inhibition of the BRCA1 gene has failed to show clinical value, thus alternative methods to target its expression must be found. HDAC inhibitors have shown some promise as single agent therapies for EOC, though they may be most useful as an addition to standard therapies. There is also evidence that HDAC inhibitors could function in part via targeting BRCA1 expression. In this study we demonstrate that the addition of an inhibitor of HDAC activity, M344, potentiates cisplatin treatment via targeting of the BRCA1 tumour suppressor gene.

Variable levels of BRCA1 mRNA and protein were observed in the ovarian and breast cancer cell lines we analyzed. This is consistent with the range of expression levels we and others have observed in ovarian and breast tumour specimens (2, 3, 32, 33). As anticipated the HCC1937 breast carcinoma cells did not express any BRCA1 protein, as they possess a germline BRCA1 mutation resulting in protein truncation (29). They did have detectable levels of BRCA1 mRNA, though it was lower than the other breast cancer cell lines examined, which is in keeping with previous observations that tumours from germline mutation carriers express mRNA levels significantly lower than those seen in sporadic tumours (34).

Studies conducted both *in vitro* and *in vivo* have demonstrated some cytotoxic efficacy of HDACi in ovarian cancer models (26, 28, 35-39). Recent clinical studies however, have shown minimal value of these compounds as single agent treatments in this disease (40). HDACi have also been shown, in ovarian and other cell types, to enhance the effect of radiotherapy and some types of chemotherapy (27, 41, 42); however the molecular mechanism behind this effect is not well understood. We have previously shown that co-treatment of the ovarian cancer cells A2780s/cp with the HDACi M344 increased sensitivity to cisplatin (3) and here we show that co-treatment of ovarian and breast cancer cell lines with M344 and cisplatin was more effective than treatment with either agent alone in the cells which displayed higher levels of BRCA1 protein and mRNA. Both the breast (T-47D) and the ovarian (A2780s) lines with the highest BRCA1 levels displayed the greatest potentiation of cisplatin cytotoxicity with the addition of M344. No effect was observed in the BRCA1-deficient HCC1937 cells and little to no effect in the low-expressing OVCAR4, SKOV3, and BT549 cells. Cells with low levels of BRCA1

were, in general, more sensitive to cisplatin than those with higher levels, consistent with the clinical observation that BRCA1 levels in tumours are predictive of chemoresponse in ovarian cancer (1, 3). Thus, co-treatment with an HDACi was able to potentiate the effect of cisplatin in breast and ovarian cancer cells that displayed minimal response to cisplatin treatment alone, which could be a result of targeting of BRCA1 by M344. There are currently clinical trials recruiting ovarian cancer patients to assess combinatorial treatment with carboplatin and SAHA. It would be of great interest to observe whether tumour BRCA1 levels influence outcome in this treatment setting.

In order to determine if treatment with M344 was affecting BRCA1 mRNA and protein levels, ovarian and breast cancer cell lines were treated with M344 alone or in combination with cisplatin and expression levels were assessed via quantitative RT-PCR and western blotting. BRCA1 protein levels were found to be decreased by treatment with M344 alone in the entire cohort of cell lines examined. The effect was more pronounced in cells with higher BRCA1 protein levels. Treatment with cisplatin, as well as other DNA-damaging agents, has been found to result in an increase in BRCA1 protein, reflective of the engagement of the DNA damage response triggered by the DSBs caused by this treatment (43, 44). Treatment with M344 in conjunction with cisplatin also reduced BRCA1 protein levels in all of the cell lines assessed, thus counteracting the effect of cisplatin treatment alone. At the mRNA level, treatment with M344 alone, at the highest concentration examined, resulted in a significant decrease in BRCA1 expression in all of the cell lines examined. Treatment with M344 in combination with cisplatin resulted in a decrease in BRCA1 mRNA expression as compared to treatment with cisplatin alone in all of the breast cancer cell lines. This decrease was not statistically

significant in the BT549 cells, which displayed low baseline levels of BRCA1, however there was a significant decrease observed in the BRCA1-mutant HCC1937 cells. In the ovarian cancer cell lines, a significant decrease was observed in all of the lines, with the exception of the A2780s cells. A2780s cells have wildtype p53 status and are exquisitely sensitive to cisplatin treatment, whereas A2780cp and SKOV3 cells are mutant for p53 and minimally sensitive to cisplatin. OVCAR4 cells are quite sensitive to cisplatin treatment, but are also mutant for p53 (45). Thus, in ovarian cancer cells, p53 status may be an important determinant of the ability of an HDAC inhibitor to target BRCA1 in the presence of DNA-damaging agents, and is a phenomenon that warrants further investigation.

The BRCA1 tumour suppressor gene plays a major role in the detection and repair of DNA damage (6, 7, 46). Without functional BRCA1 activity, cells demonstrate an increase in irreparable DNA double-strand breaks in response to DNA-damaging agents such as chemotherapeutics due to a shift from the HR pathway to the more error prone NHEJ pathway (32). A hallmark of DNA DSBs is the formation of γH2A.X foci, resulting from the rapid phosphorylation of γH2A.X at sites of DSBs (47). The extent to which foci formation is observed can be used to assess levels of DNA damage. When we examined A2780s cells treated with M344 alone or in combination with cisplatin for γH2A.X foci formation via direct immunofluorescence as well as by sorting via flow cytometry, minimal foci formation was observed with M344 treatment alone, indicating that treatment with this HDAC inhibitor as a single agent was not sufficient to induce significant amounts of DNA damage. Treatment with M344 in addition to cisplatin resulted in an increase in the level of DNA damage as compared to cisplatin alone,

demonstrating that the addition of M344 to cisplatin treatment enhances levels of DNA damage. As BRCA1 levels are being reduced by this treatment, this increase in DNA damage is likely a result of a failure in damage repair resulting from decreased BRCA1 expression, though the precise mechanism behind this warrants further investigation.

We have shown that treatment of ovarian and breast cancer cells with the HDACi M344 enhance the cytotoxicity of cisplatin therapy, particularly in cells with higher baseline expression of the tumour suppressor BRCA1. We have also demonstrated that this treatment combination results in a decrease in BRCA1 expression levels, indicating this HDACi may effectively target BRCA1. This is significant as lower BRCA1 levels have been shown to be a positive predictive marker of progression-free survival and overall survival in ovarian cancer. Thus co-treatment with an HDACi and a platinum-based agent may provide an effective novel treatment for ovarian cancer via the targeting of BRCA1.

Figure 1: **A)** Western blot analysis of basal expression levels of BRCA1 protein in a panel of cell lines. Actin was used as a loading control. Numbers indicate protein densitometry readings with MCF7 used as the calibrator and set to 1.0. **B)** Basal levels of BRCA1 mRNA analyzed by real time quantitative RT-PCR. Fold changes were calculated following normalization to GAPDH levels and expressed as the mean +/- SEM.

Figure 2: MTT viability assays comparing the responses of a panel of cell lines to 0-8 μg/ml cisplatin alone (■) or with co-administration of 1 μM M344 (▲). Cell viability was assayed with the activity of untreated cells taken to be 100%. Numbers represent the mean +/- SEM of where n= 3 in triplicate. Differences between treatment with cisplatin alone versus treatment with cisplatin and M344 were analyzed using 2-way ANOVA with Bonferroni posttest. * indicates a significant difference where $P<0.05$, ** where $P<0.01$, and *** where $P<0.001$.

Figure 3: Percentage of apoptotic cells in a panel of cell lines following 24 hours of treatment with 1.0 μM M344 alone or in combination with 2 μg/ml cisplatin as assessed by flow cytometry.

Figure 4: Western blot analysis of BRCA1 in a panel of cell lines following 24 hours of treatment with 0.5, 1.0, or 5.0 μM M344 either alone or in combination with 2 μg/ml cisplatin. Numbers represent densitometry readings with untreated control used as the calibrator and set to a value of 1.0.

Figure 5: BRCA1 mRNA levels in a panel of cell lines analyzed by real time quantitative RT-PCR following 24 hours of treatment with 0.5, 1.0, or 5.0 μM M344 either alone or in combination with 2 μg/ml cisplatin. Numbers represent the mean +/- SEM of three separate experiments. Differences between treatments were analyzed using 1-way ANOVA with Bonferroni posttest. Statistically significant differences, when present, between 5 μM M344 and DMSO control or 2 μg/ml cisplatin + 5 μM M344 and 2 μg/ml cisplatin alone are indicated by * where $P<0.05$, ** where $P<0.01$, and *** where $P<0.001$.

Figure 6: **A)** Cells were subjected to treatment with DMSO control, cisplatin or M344 or both for 24hrs and then stained with a fluorescence-labelled antibody for γH2A.X **B)** Following treatment, cells were probed with an anti-γH2A.X antibody and sorted via flow cytometry.

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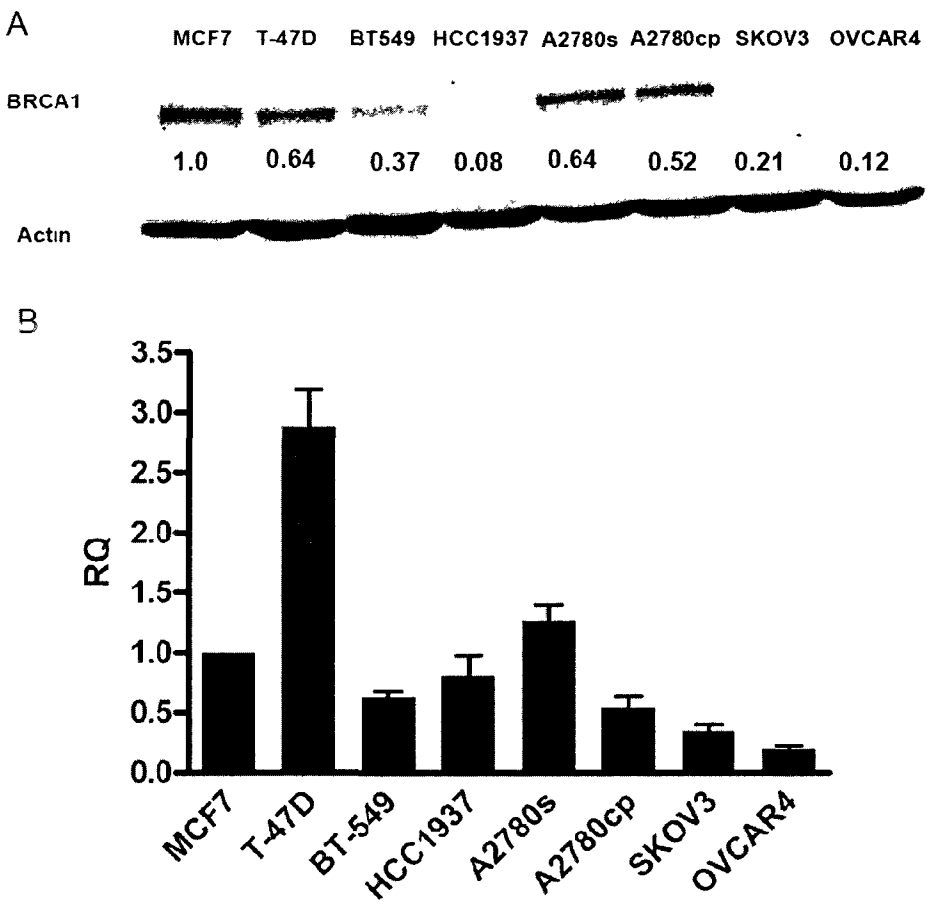
A
MCF7
T-47D
BT549
HCC1937
A2780s
A2780cp
SKOV3
OVCAR4
BRCA1
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0.64
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0.08
0.64
0.52
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Actin
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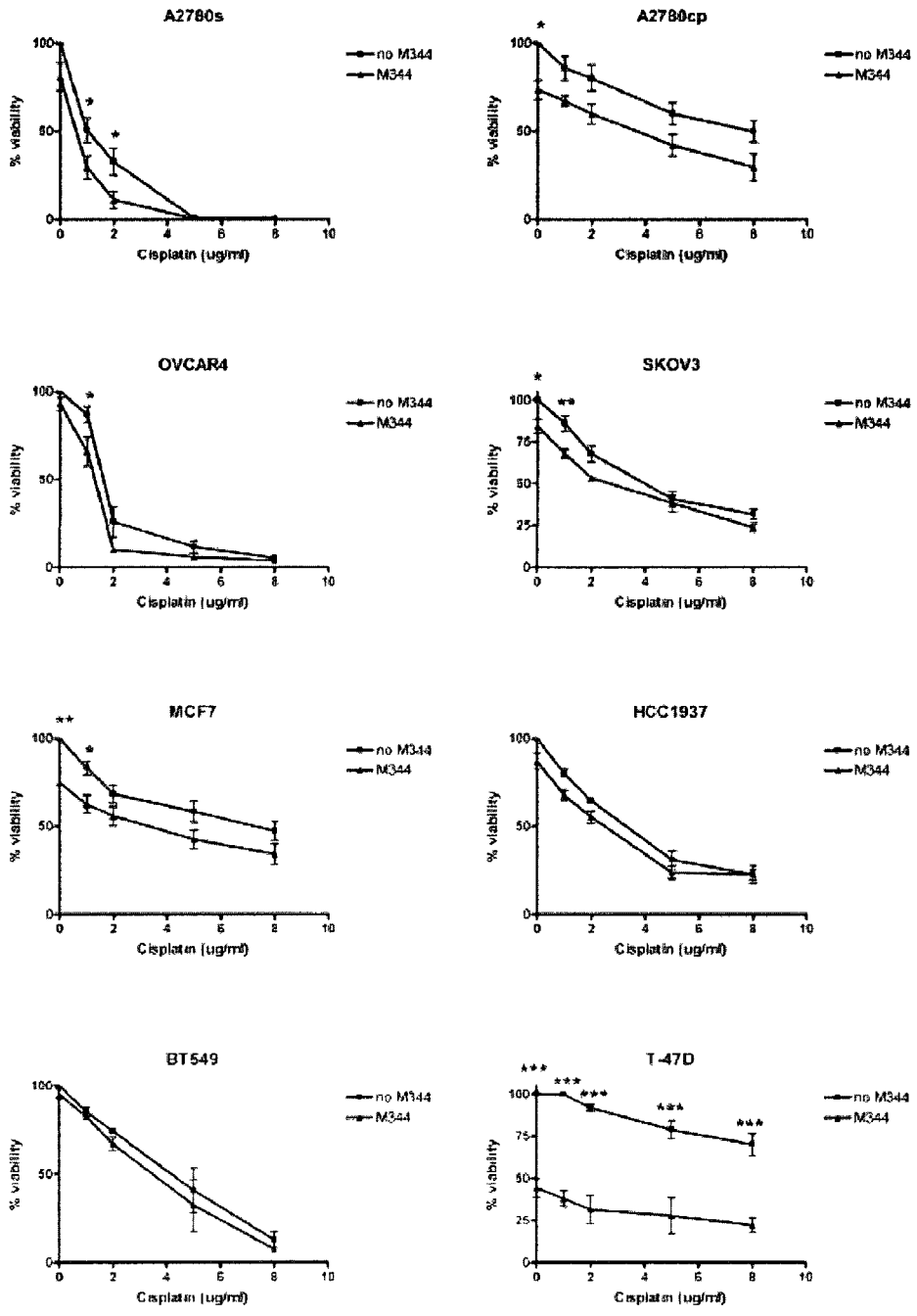
SKOV3
OVCAR4

Figure 1


A2780s
A2780cp
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BT549
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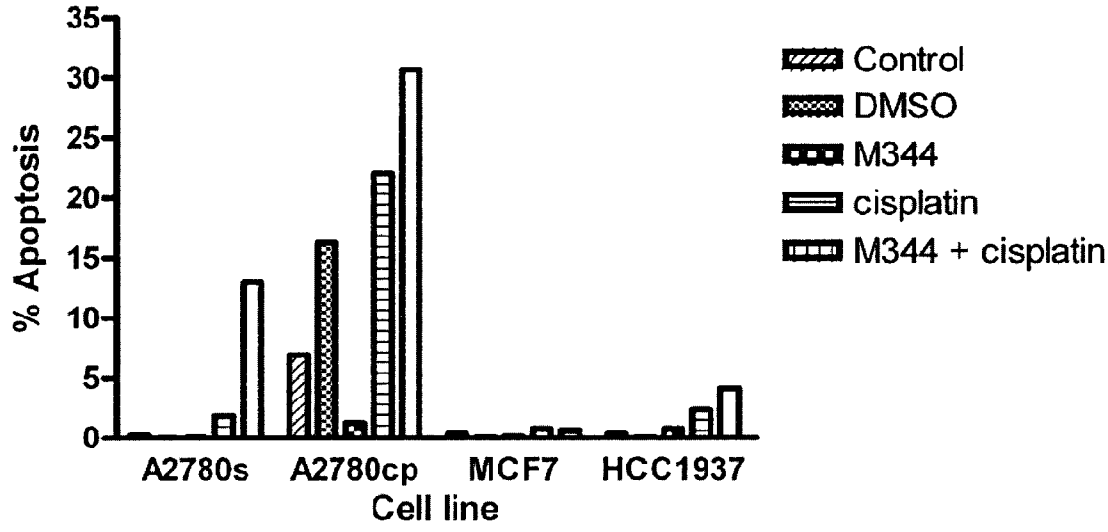
M344
% viability
Cisplatin (ug/ml)

Figure 2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30
25
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0
% Apoptosis
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MCF7
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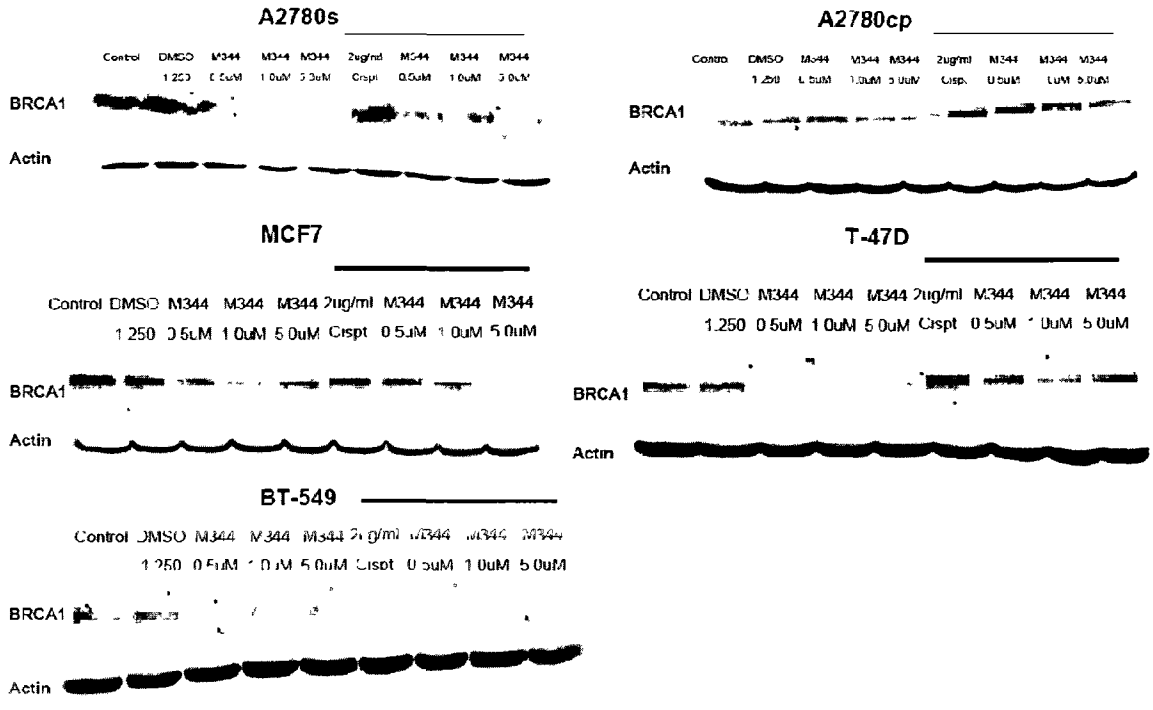
Cell line
Control
DMSO
M344
cisplatin
M344 + cisplatin

Figure 3


A2780s
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BRCA1
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MCF7
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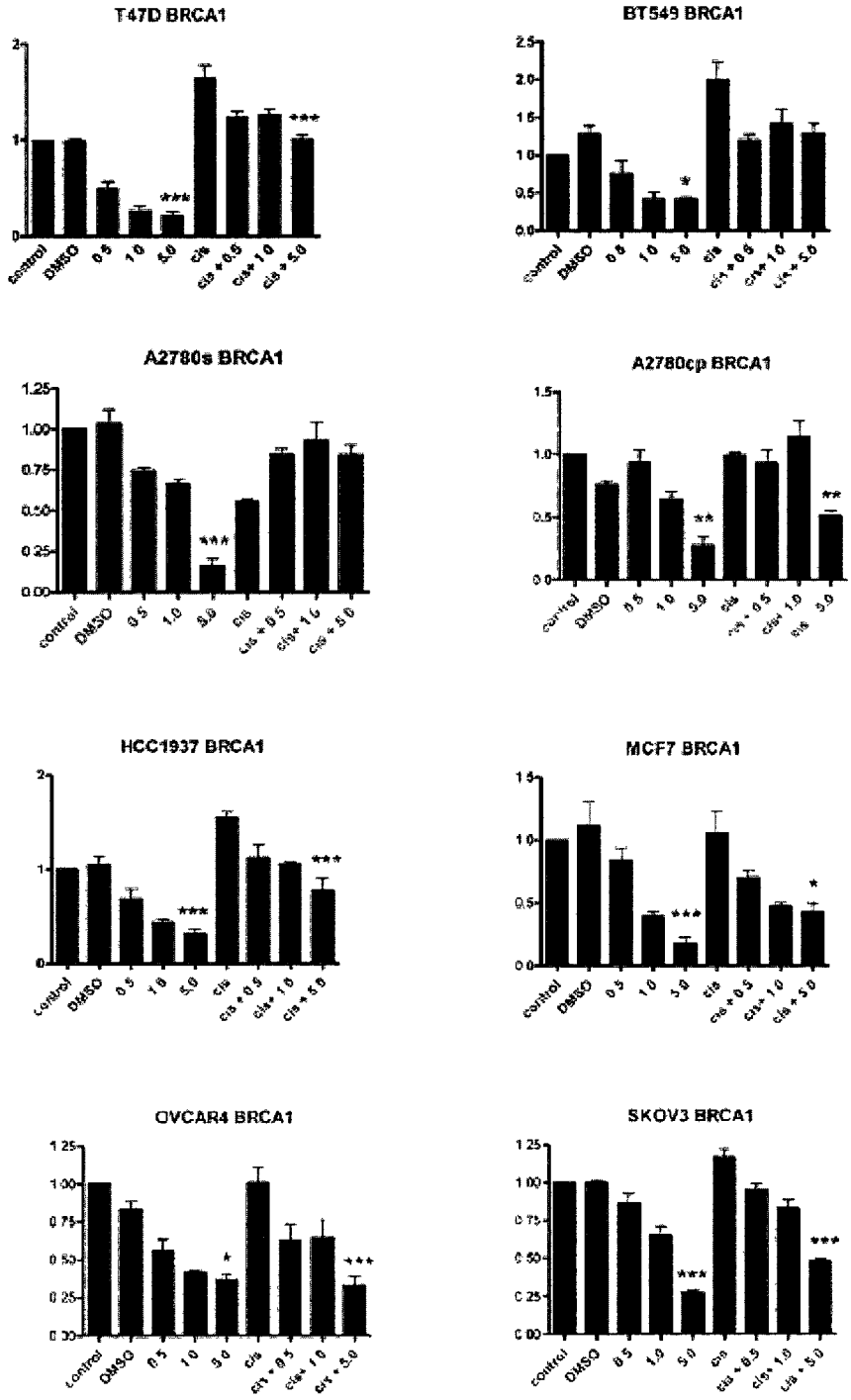
Actin

Figure 4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BT549 BRCA1
A2780s BRCA1
A2780cp BRCA1
HCC1937 BRCA1
MCF7 BRCA1
OVCAR4 BRCA1
SKOV3 BRCA1
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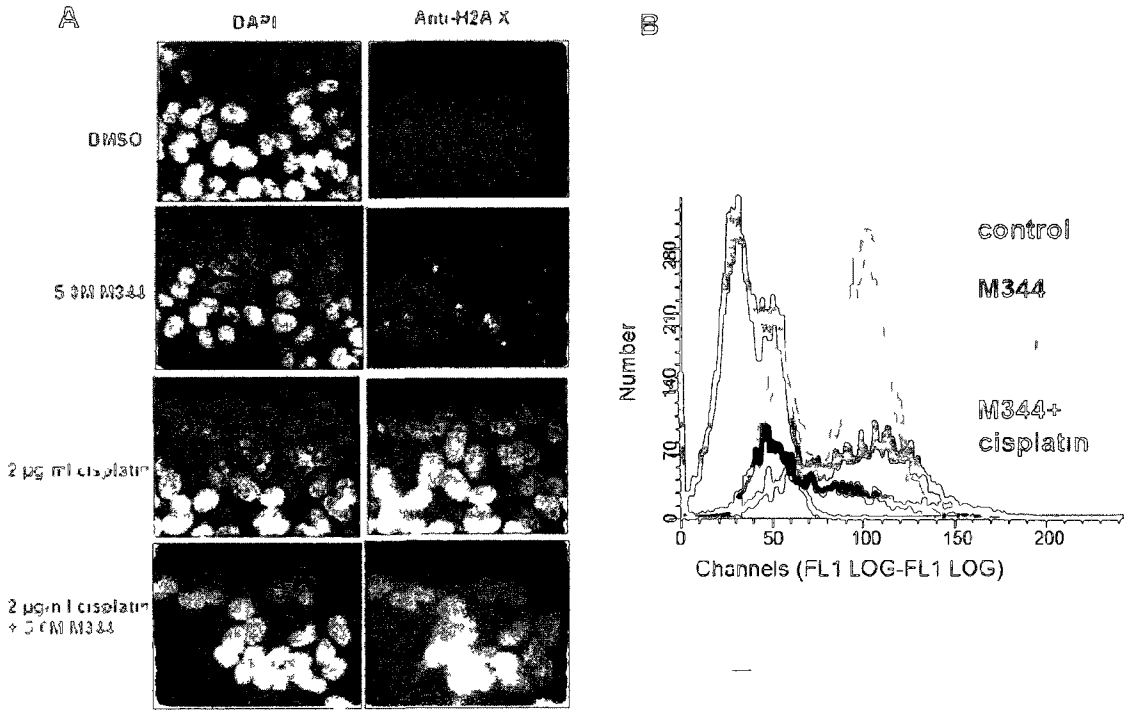
1.0
5.0
cis
cis + 0.5
cis + 1.0
cis + 5.0

Figure 5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DAPI
Anti-H2A X
DMSO
5 µM M344
2 µg ml cisplatin
2 µg ml cisplatin
+ 5 µM M344
B
control
M344
M344+
cisplatin
Number
0
70
140
210
280
0
50
100
150
200
Channels (FL1 LOG-FL1 LOG)

Figure 6

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

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

Running Title: ATF3 regulates cisplatin cytotoxicity

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Abstract

The mechanisms underlying the pro-apoptotic effect of the chemotherapeutic agent, cisplatin, are largely undefined. Understanding the mechanisms regulating cisplatin cytotoxicity may uncover strategies to enhance the efficacy of this important therapeutic agent. This study evaluates the role of Activating Transcription Factor 3 (ATF3) as a mediator of cisplatin-induced cytotoxicity. Cytotoxic doses of cisplatin and carboplatin treatments consistently induced ATF3 expression in five tumour derived cell lines. Characterization of this induction revealed a p53, BRCA1, and integrated stress response (ISR), independent mechanism, all previously implicated in stress mediated ATF3 induction. Analysis of MAPKinase pathway involvement in ATF3 induction by cisplatin revealed a MAPKinase dependent mechanism. Cisplatin treatment combined with specific inhibitors to each MAPKinase pathway (JNK, ERK and p38), resulted in decreased ATF3 induction at the protein level. P38 pathway inhibition led to decreased ATF3 mRNA expression and a reduction in the cytotoxic effects of cisplatin as measured by MTT cell viability assay. In A549 lung carcinoma cells, targeting ATF3 with specific shRNAs also attenuated the cytotoxic effects of cisplatin. Similarly, ATF3 -/- MEFs were shown to be less sensitive to cisplatin induced cytotoxicity as compared with ATF3+/+ MEFs. This study identifies cisplatin as a MAPKinase pathway dependent inducer of ATF3, whose expression influences cisplatin's cytotoxic effects.

Abbreviations: ATF, activating transcription factor; cisplatin , cis-Diamminedichloroplatinum(II), ISR, integrated stress response; MAPKinase, mitogen-activated protein kinase; MEFs, murine embryonic fibroblasts; cisplatin , cis-Diamminedichloroplatinum(II)

Introduction

cis-Diamminedichloroplatinum(II) (cisplatin) is among the most active anti-tumour agent used in human chemotherapy. Cisplatin and its derivative, carboplatin, are widely used agents in various tumour types including lung and ovarian cancers [1]. Acquired resistance and toxicities associated with treatment are major impediments inhibiting their efficacy [2]. Understanding the mechanisms regulating tumour cell cytotoxicity may uncover novel therapeutic strategies to enhance the efficacy of these platinum-based chemotherapeutics. Cisplatin and carboplatin are primarily considered as DNA-damaging anticancer drugs forming different types of bi-functional adducts in reaction with cellular DNA [1]. Cisplatin and carboplatin become activated intra-cellularly by the aquation of one of two chloride leaving groups, and subsequently covalently bind to DNA, forming DNA adducts [3]. Carboplatin is a less toxic compound with a more stable leaving group than chloride which lowers toxicity, and reduces nephrotoxicity, without affecting anti-tumour efficacy [4]. The final cellular outcome of DNA adduct formation is generally apoptotic cell death, thought to occur through halting of cellular processes such as replication and transcription leading to prolonged G2 phase cell-cycle arrest and deregulation of signal transduction pathways involved in growth, differentiation, and stress responses [1, 3]. Cellular mechanisms of resistance to platinum-based chemotherapeutics are multi-factorial and contribute to severe limitation in their use in clinical practice. They include molecular events inhibiting drug-DNA interaction, such as a reduction in cisplatin accumulation inside cancer cells or inactivation by thiol-containing species [2]. Other important mechanisms acting downstream to the initial reaction of cisplatin with DNA include an increase in adduct

repair and a decrease in induction of apoptosis [2]. Although DNA is the primary target of cisplatin and carboplatin activity, there still remains gaps in our understanding of the process that translates cisplatin induced DNA damage into its therapeutically beneficial process of apoptosis. Two significant cellular pathways have been demonstrated to play key roles in platin-induced apoptosis/cytotoxicity, the mitogen activated protein kinase cascades (MAPKinase) and the tumor suppressor p53 [5, 6]. An understanding of the mode of action is indeed desirable in refining therapeutic approaches that further enhance the anti-tumour activity of platinum based chemotherapeutics.

Activating transcription factor 3 (ATF3) is a member of the basic region-leucine zipper proteins originally identified for their ability to bind the cAMP responsive element (ATF/CRE) site (TGACGTCA) [7] While ATF3 mRNA and protein levels are not detectable under basal conditions in most cells, a large body of evidence shows that ATF3 is induced by a wide variety of stress causing agents including hypoxia, metabolic stress and DNA damage [8]. ATF3 is also induced in times of physiological stress such as liver regeneration [9], brain seizure [10], ischemia-reperfusion of the heart [11] and nerve damage [12, 13]. ATF3 has been demonstrated to play a role in apoptosis and proliferation, two cellular processes critical for cancer progression [14-17]. ATF3 can either promote or suppress these processes For example, over expression of ATF3 in the sense orientation in colorectal cancer cells led to decreased focus formation in vitro and reduced the size of mouse tumour xenografts in vivo [14]. Divergence in function of ATF3 between a pro-and anti-apoptotic factor in cancer models is likely dependent on both cellular model and state of malignancy [17, 18] ATF3 is also a member of the Activating Protein–1 (AP-1) transcription factors which consist of homodimers and

heterodimers of the basic region-leucine zipper proteins that belong to the Jun (c-Jun, v-Jun, JunB, JunD), Fos (c-Fos, v-Fos, FosB, Fra1, Fra2) and the related activating transcription factor (ATF2, ATF3/LRF1, B-ATF) subfamilies [19] Activation of ATF3 by a wide array of stress signalling pathways have been demonstrated including DNA repair pathway components p53 [20, 21] and potentially BRCA1 [15, 22], the integrated stress response that is principally activated by hypoxia and metabolic stress [23], and the stress induced MAPKinase cascades (SAPK/JNK, p38 and ERK) [24, 25] Of interest, p53 and MAPKinase cascades have also been shown to play roles in regulating cisplatin-induced cytotoxicity However the downstream effectors that regulate cisplatin-induced cytotoxicity have not been established

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

Material and Methods

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

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay. In a 96-well flat-bottomed plate (Nunc Naperville, IL) ~5 000 cells/150 µL of cell suspension were used to seed each well The cells were incubated overnight to allow for cell attachment and recovery Cells were treated with indicated drugs and incubated for 48 h at 37°C Following treatment, 42 uL of a 5 mg mL solution in PBS of the MTT tetrazolium substrate (Sigma) was added to each well and incubated for ~ 20 min at 37°C

The resulting violet formazan precipitate was solubilized by the addition of 82 µL of a 0 01 mol/L HCl/10% SDS (Sigma) solution, and allowed to further incubate at 37°C overnight. The plates were then analyzed on an MRX Microplate Reader from Dynex Technologies (West Sussex, United Kingdom) at 570 nm to determine the absorbance of the samples

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

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

agent Background Sniper (Biocare Medical; Brampton, ON, Canada) for 20 min at room temperature, incubated with ATF3 antibody (1:200 dilution in DaVinci universal diluent (Biocare Medical)) for 1 h at room temperature, rinsed with TBS for 5 min, incubated with universal mouse probe (Biocare Medical Mach 4 universal polymer detection kit) for 5 min, rinsed with TBS for 5 min, incubated with Rabbit HRP Polymere (Biocare Medical Mach 4 universal polymer detection kit) for 10 min at room temperature, rinsed with TBS, and developed for 5min with DAB RTU (Biocare Medical Betazoid DAB chromatogen kit) and rinsed with water. Slides were counterstained in hematoxylin for 1 min, washed in running water, 0.2% HCl in 70% alcohol for 5 dips, washed in running water 1 min. dipped once in 2% aqueous saturated lithium carbonate, washed in running water 5 min. dehydrated in absolute alcohol, cleared in toluene and mounted on cover slips with permount

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

Design and expression of small hairpin RNAs. The two 19mer sequences targeting ATF3 mRNA are; #1-5'-GCCAAAGAATATTCCATTT-3' and #2- 5'-GGGAGGGCCTGCAGTGATT-3' to pSuper vector from Oligoengine small hairpin RNA (shRNA) (#1. nucleotides 1524-1542. GenBank accession number

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

Western Blot Analysis Cells plated at 0 7 X 10^6 , 60 mm dish were allowed to grow overnight and treated with indicated drug for 24 h Protein samples were collected in RIPA buffer (50 mM Tris-CL pH 7 5 150 mM sodium chloride, 1mM EDTA 1% Triton-X-100 0 25 % sodium deoxycholate, 0 1% SDS) containing 50 mM sodium fluoride, 1mM sodium orthovanadate 10 mM β-glycerolphosphate and 1X Protease Inhibitor Cocktail (Sigma-Aldrich. St Louis MO) Protein concentrations were assayed using Bio-Rad Protein Assay (Mississauga. Ontario Canada) and a Biomate 3 Spectrophotometer (Thermo Fisher Scientific Waltham. MA) Protein extracts

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

RT-RNA isolation and RT-PCR. MCF-7 cells plated at 0 8 X 10^6 cells per 10 cm dish were incubated at 37°C overnight. The next day cells were treated with cisplatin (in the absence or presence of SB203580) for 24 h. Total RNA was extracted from cell samples using the RNeasy1 kit (Qiagen, MD). RNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). One microgram of total RNA was reverse-transcribed to complementary DNA for quantitative, real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described [26] The

Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City,CA) was used to detect amplification. A real-time PCR reaction was carried out in a total volume of 25 µl that contained 2.5 µl of synthesized cDNA (42 ng), 1.25 µl of Taq Man Gene Expression Assay Primer/Probe (20X) (Applied Biosystems, ATF3, HS00231069), 12.5 µl of Taq Man Universal PCR Master Mix (2X) (Applied Biosystems, 4304437) and 8.75 µl of RNase-free water for ATF3 expression. The endogenous control for ATF3 was the housekeeping gene, human GAPDH (20X) (Applied Biosystems, HS4333764-F). Amplification conditions were 95°C for 5 min, 40 PCR cycles at 95°C for 15 sec and 60°C for 1 min. Three independent experiments were performed to determine the average gene expression and standard deviation.

Results

Cisplatin and carboplatin cytotoxicity is associated with ATF3 induction

We analyzed the cytotoxic effects of cisplatin and carboplatin treatment on a number of human tumour cell lines, MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), SKOV-3 (ovarian carcinoma), PC3 (prostate carcinoma) and A2780-cp (ovarian carcinoma), using the MTT cell viability assay and flow cytometry (Figure 1) In A549, PC3 and A2780-cp cell lines higher doses of cisplatin lead to 100 % cytotoxicity (Figure 1A) whereas the MCF-7 and SKOV-3 cell lines appeared more resistant as determined by the MTT cell viability assay Resistance to the cytotoxic effects of cisplatin in the MCF-7 and SKOV-3 cell lines was evidenced by a plateau patterning at higher doses of the treatment a pattern previously suggested to be related to deficiency in pro-apoptotic factors [2] (Figure 1A) Indeed, the MCF-7 cell line is caspase-3 deficient [27] Carboplatin also induced cell cytotoxicity in all cell lines and similar to cisplatin. MCF-7 and SKOV-3 displayed the greatest resistance to the cytotoxic effects (Figure 1B) We further employed flow cytometry analyses to determine the potential for apoptosis induction by the platinum-based chemotherapeutics in the A549 and SKOV-3 cell lines Apoptosis was visualized as a sub-2N peak that identifies apoptotic bodies resulting from cellular fragmentation [28. 29] Cisplatin treatment resulted in 10 19 % and 8 42% of cells in the sub-2N fraction in A549 and SKOV-3 cell lines respectively (Figure 1C) Carboplatin treatment resulted in 23 44 % and 11 33% of cells in sub-2N in A549 and SKOV-3 cell lines. respectively (data not shown)

Previously our lab had identified lovastatin. a potent inhibitor of mevalonate synthesis as an inducer of the ISR pathway and subsequent mediator of lovastatin-

induced apoptosis [30] Downstream effectors of the ISR pathway activated by lovastatin included members of the Activating Transcription Factor (ATF) family, ATF4 and ATF3 A role for the stress inducible gene, ATF3, in tumourgenesis has been demonstrated and can act as either a tumour suppressor or oncogene depending on cell context. Since various stress pathways that induce ATF3 expression have also been shown to regulate cytotoxicity, we first evaluated the potential of cisplatin and carboplatin to affect ATF3 expression Indeed we found that ATF3 was significantly induced at the protein level when treated for 24 h with cytotoxic concentrations of cisplatin (10μg/ml) and carboplatin (100 and 500 μM) in a panel of human cancer cell lines (Figure 2A) These higher concentrations of drug treatment induced significant cytotoxicity at 48 h treatments as demonstrated by MTT assay analysis (Figure 1A) Immunocytochemical analysis of ATF3 expression in cisplatin (8μg/ml) treated MCF-7 and PC3 cells for 24 h showed that while untreated cells did not express ATF3, a significant proportion of cisplatin treated cells showed expression and nuclear localization of this transcription factor (Figure 2B) Time course analysis of ATF3 induction by cis- and carbo-platin revealed maximal induction levels occurring at 12 and 24 h in the MCF-7 cell line (Figure 2C) Furthermore it was demonstrated that cisplatin could significantly induce the levels of ATF3 mRNA (Figure 2D) In summary, ATF3 is highly induced at the protein and mRNA level by cisplatin in human cancer cell lines

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

ATF3 mRNA and protein levels are readily induced by a wide range of stress causing agents [8] The mechanism(s) of stress induced ATF3 has been previously well

documented (Figure 3A) and in this study, we evaluated these regulatory mechanisms with respect to cisplatin induction of ATF3. Since the tumour suppressor p53 had been previously implicated in ATF3 regulation [20, 21] we determined its role in cisplatin induction of ATF3. ATF3 was induced by cisplatin in the p53 functionally null cell lines SKOV-3 and PC3, which suggested a p53-independent mechanism (Figure 1A). To further investigate a possible regulatory role, we virally expressed p53 in the PC3 cell line, treated the cells with cisplatin or taxol, a microtubule depolarizing agent, and determined ATF3 expression levels. ATF3 induction by cisplatin was unchanged between LacZ vector control and p53 containing viral infection under cisplatin treatment further confirming a p53-independent mechanism (Figure 3B, middle panel). Taxol treatment had no effect on ATF3 expression levels (Figure 3B, bottom panel). We next looked at the possible involvement of the DNA damage response factor, BRCA1, in the regulation of ATF3 induction by platinum-based chemodrugs since previous reports suggested that BRCA1 could transcriptionally regulate ATF3 expression [15, 22]. In order to determine whether BRCA1 played a role in ATF3 induction by cis-or carboplatin, ATF3 induction was contrasted in cell lines expressing and null for BRCA1, MCF-7 and 1937, respectively. As shown in Figure 3C, no difference in ATF3 induction levels was observed between the two cell lines with either treatment suggesting that induction of ATF3 by the chemodrugs is independent of BRCA1 expression. Next we evaluated the role of the ISR pathway in mediating ATF3 induction since ATF3 is a downstream effector of the pathway [23]. We tested the ability of cisplatin and carboplatin to induce ATF3 expression in immortalized heterozygous or ATF4 null MEFs, the upstream inducer of ATF3 expression in the ISR pathway. Figure 3D

demonstrates the absence of ATF4 had no affect on ATF3 induced by cis- and carboplatin suggesting an ISR independent mechanism as well

MAPKinase pathways regulate ATF3 induction by cisplatin.

Recent characterization of ATF3 induction by anisomycin revealed a MAPKinase dependent mechanism [25], therefore, we investigated the individual MAPKinase pathways for potential regulation of ATF3 expression by platinum-based chemodrugs. First we determined whether the pathways were activated under the drug treatments in the MCF-7 cell line Time course analysis of JNK pathway activation following treatment with platinum-based chemodrugs as measured by the phosphorylation status of c-Jun, a downstream effector of the JNK pathway cascade revealed a slight increase in phospho-c-Jun at 12 and 24 h which coincided with maximal ATF3 induction by both cisplatin and carboplatin (Figure 4A. left panel) To determine the role of the JNK pathway in ATF3 induction by cisplatin and carboplatin, MCF-7 cells were treated with cis- or carbo-platin for 24 h in the presence of a JNK specific inhibitor, JNK Inhibitor II SP600125 (SP), which revealed effective blockage of the pathway as measured by phospho-c-Jun levels (Figure 4A. right panel) ATF3 induction levels were found to be reduced under cisplatin treatment in the presence of JNK inhibitor but not with carboplatin, suggesting a role for the JNK pathway in mediating ATF3 induction by cisplatin (Figure 4A. right panel) Next we evaluated the role of the ERK pathway in ATF3 induction by cis- and carbo- platin Time course treatment with chemodrugs revealed activation of the ERK pathway as measured by the phosphorylation status of ERK at 4h under cisplatin and 4 and 8h under carboplatin treatment (Figure 4B. left

panel). Phosphorylated ERK was not detected at 12 and 24 hrs under either treatment (Figure 4A, left panel). To determine the role of the ERK pathway in ATF3 induction by cisplatin and carboplatin, MCF-7 cells were treated with cis- or carbo-platin in the presence of the specific inhibitor to the ERK pathway, UO126, which was effective in blocking of the pathway as measured by phospho-ERK levels (Figure 4B, right panel). Treatment with ERK inhibitor in the presence of cis- and carbo-platin revealed a dose dependent decrease in ATF3 expression levels suggesting a role for the pathway in mediating induction by the chemodrugs (Figure 4B, right panel). Lastly, we determined the activation of the p38 pathway in MCF-7 cells following treatment with cis- or carbo-platin at 9 and 24 h time points. Indeed it was observed that the pathway was active at these time point as measured by the phosphorylation status of p38, which correlated with ATF3 induction levels (Figure 4C, left panel) To investigate the role of p38 pathway in the induction of ATF3 by cis- and carbo-platin MCF-7 cells were treated with chemodrugs in the presence of the specific p38 inhibitor, SB203580 (SB), for 24 h. This p38 inhibitor was shown to effectively block the pathway as measured by the phosphorylation status of the Heat Shock Protein 27 (hsp27), a downstream target of p38 (Figure 4C, right panel). ATF3 expression levels were also shown to be decreased by the inhibitor in a dose dependent manner suggesting a regulatory role of the p38 pathway in chemodrug induction of ATF3 (Figure 4C, right panel). We next characterized the involvement of the MAPKinase pathways in the tumour derived cells lines SKOV-3, PC3, and A459 and found that indeed all inhibitors against the three MAPKinase pathways had variable degrees of inhibition of ATF3 induction by cisplatin implicating all three pathways in the mechanistic induction of ATF3 by cisplatin (Figure 5A).

Interestingly, the pattern of reduced ATF3 induction by cisplatin in the presence of MAPKinase inhibitors was consistent between all four cancer cell lines with the p38 pathway inhibitor showing the greatest inhibition of ATF3 induction and the ERK pathway inhibitor showing the least (Figure 5A) We also showed that the p38 inhibitor could down-regulate cisplatin induction of ATF3 mRNA levels by approximately 5-fold (Figure 5B) Taken together these results identify the MAPKinase pathways as regulators of ATF3 induction by platinum-based cytotoxic drugs

ATF3 regulates, in part, the cytotoxic effects of cisplatin

Since ATF3 has been previously shown to play a pro-apoptotic role in cancer models, we investigated the role of ATF3 induction by cisplatin in regulating the cytotoxic effects of cisplatin. Treating A549 cells with increasing concentrations of cisplatin in the presence or absence of SB203580 (10μM) the cytotoxic effects of cisplatin was observed to be attenuated by approximately 20 % as measured by MTT assay (Figure 6A) Similar results were obtained in the PC3 cell line (data not shown) Inhibition of ATF3 expression levels under cisplatin treatment in the presence of p38 inhibitor was also observed in the A549 cell line (Figure 6A, bottom inset) The cleavage status of Parp, a marker of apoptosis, was determined in the A549 cell line following treatment with cisplatin in the presence of MAPKinase inhibitors Indeed, PARP cleavage induced by cisplatin was reduced in the presence of MAPKinase inhibitors to all three pathways (Figure 6A, top inset) To further demonstrate ATF3 's role as a factor in the cytotoxic affects of cisplatin, stable expression of shRNA against two separate areas of ATF3-mRNA and GFP a negative control, were employed in the A549 cell line Cells

expressing both shATF3 and treated with cisplatin showed a significant decrease in ATF3 expression as compared with the GFP control (Figure 6B, inset) MTT analysis of the three cell lines treated with a range of cisplatin concentrations revealed attenuation of the cytotoxic effects of the drug in the shATF3 cell lines as compared with GFP control (Figure 6B) Lastly, we contrasted the cytotoxic effects of increasing cisplatin treatments on MEF cells expressing and knocked out for ATF3 ATF3 (+/+) MEFs were more sensitive to cytotoxic effects of cisplatin as compared with ATF3 (-/-) MEFs (Figure 6C) Cisplatin treatment induced ATF3 expression in the ATF3 (+/+) MEFs but not in the ATF3 (-/-) MEFs (Figure 6C. inset) The differences in cytotoxic effects of cisplatin observed in ATF3 (+/+) and (-/-) MEFs were contrasted in MEFs (+/+) and (-/-) for the ATF2 family member No differences in the effect of cisplatin cytotoxicity was observed between ATF2 (+/+) and ATF2 (-/-) MEFs (Figure 6D) Taken together this study provides evidence that the cytotoxic effects invoked by cisplatin can in part be correlated to the drug s ability to induce ATF3 expression as regulated by MAPKinase pathways

Discussion

In summary, this study has identified the platinum-based chemocytotoxic drug, cisplatin, as an inducer of the stress inducible gene, ATF3, at both the mRNA and protein level. Through elimination of potential ATF3 regulatory mechanisms namely, p53, BRCA1, and ISR, we identified ATF3 induction by cisplatin as regulated by the MAPKinase pathways JNK, ERK and p38. Inhibition of the MAPKinase pathway with the p38 inhibitor SB203580 in cisplatin treated cells resulted in the greatest decrease in ATF3 induction at the protein level in all of the human cancer cells analyzed. Increased ATF3 expression was associated with cisplatin-induced cytotoxicity as evidenced by attenuation of ATF3 expression and cytotoxicity with treatment of the p38 pathway inhibitor and in shATF3 knockdown cells compared with control. Furthermore, ATF3 -/- MEFs were more resistant to cisplatin cytotoxicity as compared with ATF3 +/+ MEFs. Taken together these results provide strong evidence that ATF3 is a regulator of platinum-based chemotherapeutic induced cytotoxicity. Identifying ATF3 as a cisplatin induced gene through MAPKinase pathway activation may have therapeutic relevance. Inducers of the MAPKinase pathways or other cell stress pathways that enhance ATF3 expression may augment the cytotoxic effects of cisplatin. For example, two agents that induce ATF3 expression through different mechanisms, salubrinal and proteosome inhibitors, have demonstrated synergistic cytotoxicity in myeloma cells and may represent a novel combinational therapeutic approach [31, 32].

It is well characterized that the commencement of the anti-cancer effects of cisplatin involves DNA adduct formation resulting in apoptotic cell death if the DNA damage cannot be adequately repaired [1]. However, the specific mechanism(s)

downstream of cisplatin induced DNA damage which leads to the apoptotic response are poorly defined. In this study we show that cisplatin and its derivative, carboplatin, could readily induce ATF3 expression. A role for ATF3 in tumourigenesis has been implicated through its ability to affect the transcription of a number of regulators of apoptosis and cell proliferation including CHOP and cyclin D1, respectively [33, 34] Depending on the cell type and the type and severity of the cell stressor ATF3 has been implicated as both a proto-oncogene or tumour suppressor For example, over-expression of ATF3 inhibited proliferation and induced cell cycle arrest in human cancer cells [15], whereas loss of ATF3 in a Ras transformed model resulted in higher proliferation rates and increased G1 to S phase transition efficiency [16] Treating our panel of human cancer cell lines with a high and low dose of cisplatin or carboplatin revealed an increase in ATF3 protein expression which was associated with the high cytotoxic doses of these drugs implicating a role for ATF3 in regulating platin-induced cytotoxicity Since ATF3 has been implicated as a biomarker for cell death in cancer models based on these results expression levels of ATF3 with respect to platin response in patients should be evaluated

Following the identification of ATF3 as a cisplatin induced gene, the major objective of this study was to systematically identify the mechanism of induction of ATF3 A number of cellular stress pathways have been shown to regulate the expression of ATF3 The DNA damage response factors p53 and BRCA1, had previously been linked to ATF3 regulation Likewise ATF3 is a known downstream effector of the ISR induced by ER, hypoxia, viral and metabolic stressors [23] This study showed that neither BRCA1 p53 nor the ISR were factors in ATF3 induction by cisplatin Instead the mechanism of induction of ATF3 by cisplatin was found to be largely MAPKinase

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

In conclusion, we determined the functional relevance of ATF3 expression in regulating cisplatin induced cytotoxicity. Recent literature has implicated the MAPKinase pathways in the regulation of stress induced ATF3 apoptosis. Similar to our results, that ribotoxic stress induction of ATF3 was shown to be mediated through the p38 pathway and ATF3 expression was shown as a pro-apoptotic factor in HeLa cells [25]. Another recent study reported that cisplatin could induce ATF3 in T98G glioblastoma cells at both the protein and mRNA level [24]. In contrast to our results, this report showed that the induction of ATF3 by cisplatin was anti-apoptotic. Discrepancies between our results and others could stem from differences in cellular model or status of cell line malignancy both of which have previously been suggested to determine ATF3's role as an anti-or pro-apoptotic factor [16, 17].

The literature has previously reported that cisplatin treatment results in the activation of MAPKinases [35]. Reports showing activation of the p38 pathway by cisplatin has been exclusively correlated with pro-apoptotic outcomes in a number of cell lines, whereas activation of the JNK and ERK pathways are correlated with both anti- and pro- death outcomes [35]. Inhibition of the p38 pathway with specific inhibitors has been previously shown to increase resistance to cisplatin [36, 37]. Likewise, reduced activation of the p38 pathway has been identified as a mechanism correlated with

cisplatin resistance [2]. Although the activation of MAPKinase pathways by cisplatin treatment has been documented the signalling pathways downstream of activation which determines cell fate are poorly understood. This study identifies ATF3, previously defined as a factor capable of influencing cellular fate, as a novel target of the MAPKinase pathways when activated by cisplatin treatment. This study suggests ATF3 induction by cisplatin may identify a novel factor responsible for mediating the established link between cisplatin induced MAPKinase pathway activation and cell cytotoxic outcomes. Whether ATF3 is directly activated by the MAPKinase pathway or is induced downstream of known MAPKinase pathway transcription factor targets remains to be determined. Defining the specific mechanism(s) responsible for the anti-tumour affects of cisplatin may lead to novel and improved therapeutic approaches.

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Figure Legends

Figure 1 Cisplatin and carboplatin treatment induce cytotoxicity and apoptosis in a panel of human cancer cell lines A Cell lines SKOV-3 MCF-7, A2780-cp, PC3 and A549 were treated with cisplatin (A) or carboplatin (B) for 48 h. and cell viability was assessed as measured by MTT activity Data is represented as a percentage of MTT activity where untreated cells were taken to be 100% Error bars are representative of six individual treated samples C Flow cytometry analysis of SKOV-3 and A549 cell lines treated with cisplatin for 48 h

Figure 2 ATF3 is induced by cis- and carbo-platin A ATF3 protein expression levels following treatment with low and high doses of cisplatin (1 and 10μg/ml) and carboplatin (10 and 500uM) in SKOV-3 MCF-7 PC3 A2780-cp and A549 cell lines B Immunocytochemistry analysis of ATF3 expression (brown) in MCF-7 and PC3 cell lines in non-treated cells (control) and cisplatin treatment for 24 h Methylene blue stain is used as the nuclear stain C Time course analysis of ATF3 expression in MCF-7 cells treated with cisplatin (10μg/ml) or carboplatin (100μM) at 4, 8 12 and 24 h time points D ATF3 mRNA quantified by RT-PCR in MCF-7 cells untreated, treated with 2 and 10μg/ml cisplatin for 24 h Error bars are representative of quantified mRNA from three independent experiments In all blots actin is used as a loading control

(*) 100 uM was used as the high dose for the A2780-cp cell line

Figure 3 ATF3 induction by cisplatin is independent of a p53 BRCA1 and ISR mechanism. A Schematic representation of the potential mechanisms involved in ATF3

induction by cisplatin. B. ATF3 detection in PC3 cells transduced with no virus (mock), LacZ vector control (LacZ), or $p53^{ad}$ (p53) containing adenovirus for 6 h following no treatment (control) (top panel), cisplatin (10μg/ml) (middle panel), or taxol (25μM) (bottom panel) for 24 h. C. ATF3 expression detected in MCF-7 and 1937 (BRCA1 null) cells untreated (control) or treated with cisplatin (1 and 10μg/ml) or carboplatin (10 and 500μM) for 24 h. D. ATF3 detection in ATF4 -/- and -/- MEFs untreated (control) or treated with cisplatin (1μg/ml) and carboplatin (10μM) for 24 h. In all blots actin is used as a loading control.

Figure 4. ATF3 induction by cisplatin is mediated by MAPKinase pathways in MCF-7 cells. A. MCF-7 cells treated with no treatment (control), anisomycin (20μM) for 1 h, and cisplatin (10μg/ml) or carboplatin (100μM) for 4, 8, 12 and 24 h time points were analyzed by western blotting for detection of ATF3, actin, phospho-c-jun (p-c-jun), total c-jun (left panel). MCF-7 cells treated with cisplatin (10μg/ml) or carboplatin (100μM) in the absence (0) or presence of JNK pathway inhibitor (SP, 25 and 50μM) for 24 h were analyzed for the detection of ATF3, actin, p-c-jun and total c-jun (right panel). B. MCF-7 cells treated with no treatment (control), anisomycin (20μM) for 1 h, and cisplatin (10μg/ml) or carboplatin (100μM) for 4, 8, 12 and 24 h time points, were analyzed by western blotting for detection of ATF3, actin, phospho-ERK (p-ERK) and total ERK (left panel). MCF-7 cells treated with cisplatin (10μg/ml) or carboplatin (100μM) in the absence (0) or presence of ERK pathway inhibitor (UO126, 10 and 25μM) for 24 h were analyzed for the detection of ATF3 and actin, and at 4 h for p-ERK and total ERK (right panel). C. MCF-7 cells untreated (control), treated with TNF-α (20ng/ml) for 15 and 30

min, and cisplatin (10μg/ml) or carboplatin (100μM) for 9 and 24 h time points, were analyzed by western blotting for detection of ATF3, actin, phospho-p38 (p-p38), and total p38 (left panel) MCF-7 cells treated with cisplatin (10μg/ml) or carboplatin (100μM) in the absence (0) or presence of p38 inhibitor, SB203580 (SB, 5 and 10μM) for 24 h were analyzed for the detection of ATF3, actin, phospho-hsp27 (p-hsp27), and total hsp27 (right panel)

Figure 5 Induction of ATF3 by cisplatin is mediated by MAPKinase pathways in a panel of human cancer cells A SKOV-3, MCF-7, PC3 and A549 cells untreated, and treated with cisplatin (10ug/ml) for 24 h in the absence (control) or presence of MAPKinase pathway inhibitors (SP600125 (50μM), UO126 (25μM), SB203580 (10μM)) and analyzed by western blotting for ATF3 and actin. B ATF3 mRNA quantified by RT-PCR in MCF-7 cells untreated, treated with cisplatin (10μg/ml) or cisplatin in the presence of SB203580 (10uM) for 24 h Error bars are representative of quantified mRNA from three independent experiments

Figure 6 ATF3 expression mediates, in part, the cytotoxic effects of cisplatin A A549 cells treated with cisplatin (0–10μg/ml) in the presence (closed squares) or absence (open circles) of SB203580 for 48 h was assessed for cell cytotoxicity as measured by MTT activity Data is represented as a percentage of MTT activity where untreated cells were taken to be 100%. Error bars are representative of triplicates of two independent experiments Western blot analysis for ATF3 actin, p-hsp27 and total hsp27 in A549 cells treated with 10μg/ml of cisplatin for 24 h in the absence (0) or presence of SB (5

and 10μM) (bottom inset). A549 cells treated with no treatment (untreated) and cisplatin in the presence of MAPKinase inhibitors (SP, UO126 and SB) and analyzed by western blotting for Parp and actin (top inset). B. A549 cells stably expressing short hairpin RNA against two separate ATF3 mRNA regions (shATF3-1 and shATF3-2) and GFP (negative control) were treated with cisplatin for 48 h and analysed for MTT activity. Western blotting analysis of ATF3 and actin in GFP, shATF3-1 and shATF3-2 expressing cell lines following 10μg/ml treatment for 24 h (inset) C. ATF3 (-/-) and (+/+) MEFs treated with cisplatin (0–8μg/ml) and analysed for MTT activity. Western blot analysis for ATF3 and actin in ATF3 (-/-) and (+/+) MEFs treated with 10μg/ml of cisplatin for 24 h (inset). Actin was used as a loading control. D. ATF2 (-/-) and (+/+) MEFs treated with cisplatin (0–8μg/ml) and analysed for MTT activity.

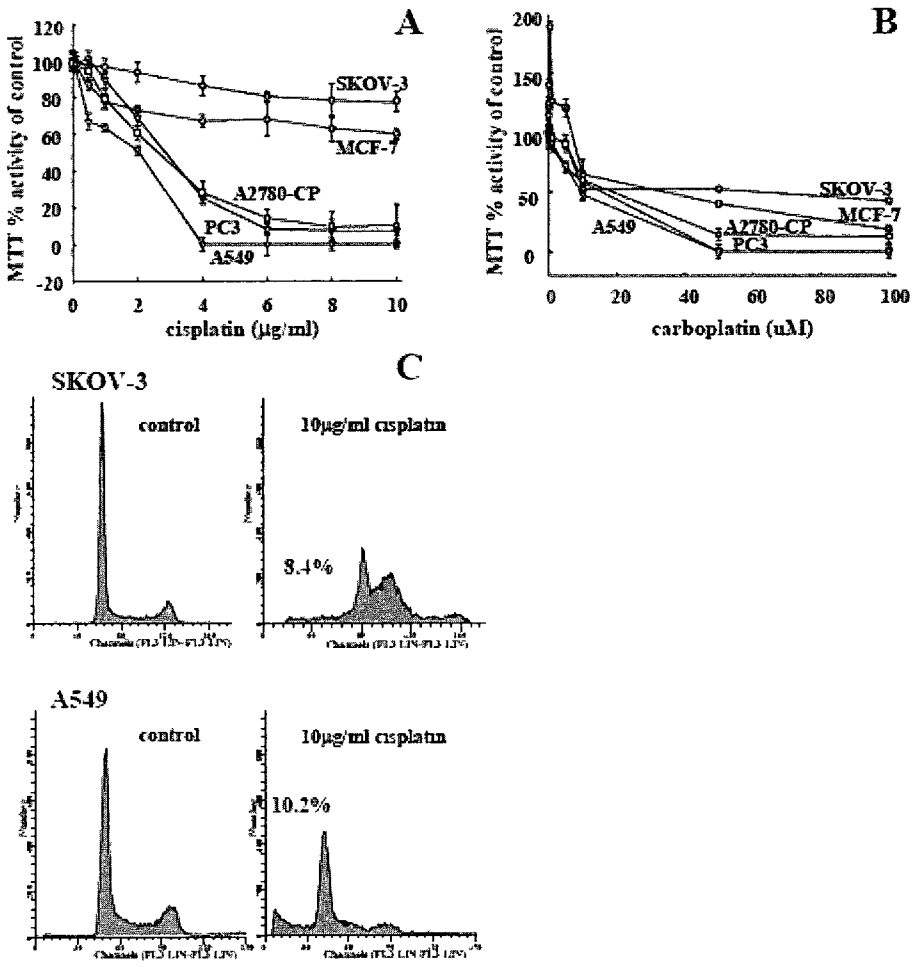

A
MTT % activity of control
cisplatin (µg/ml)
SKOV-3
MCF-7
A2780-CP
PC3
A549
B
carboplatin (uM)
C
SKOV-3
control
10µg/ml cisplatin
8.4%
A549
control
10µg/ml cisplatin
10.2%

Figure 1

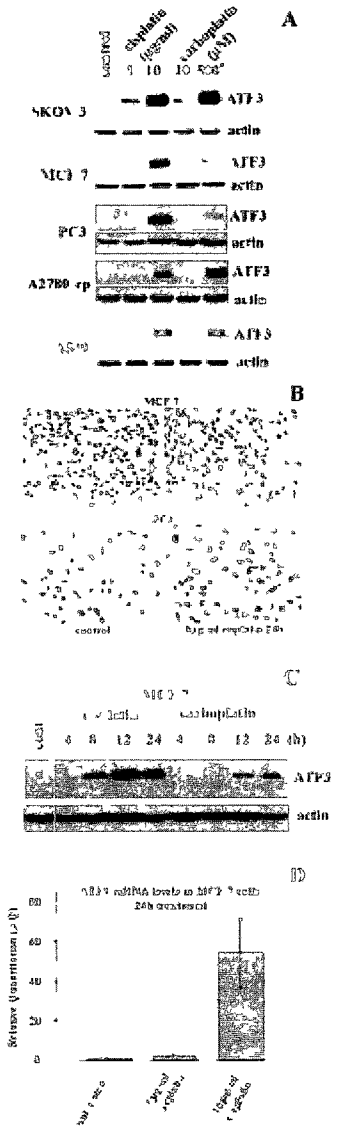

A
cisplatin (μg/ml)
carboplatin (μg/ml)
SKOV-3
ATF3
actin
MCF-7
PC3
A2780-cp
B
control
C
cisplatin
carboplatin
ATF3
actin
D

Figure 2

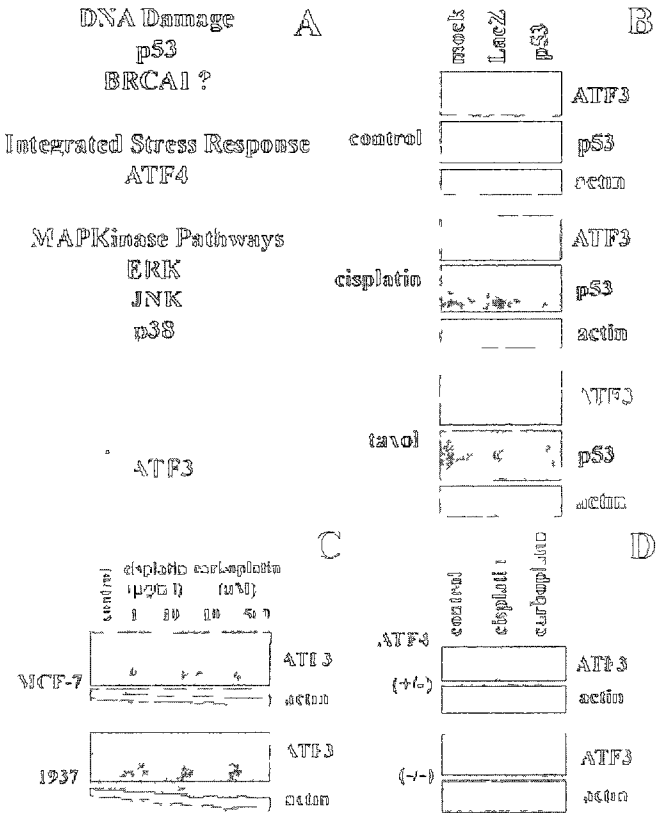

DNA Damage
p53
BRCA1 ?
Integrated Stress Response
ATF4
MAPKinase Pathways
ERK
JNK
p38
ATF3
A
B
mock
LacZ
p53
control
cisplatin
taxol
ATF3
p53
actin
C
cisplatin
carboplatin
(μM)
MCF-7
1937
D
ATF4
(+/-)
(-/-)

Figure 3

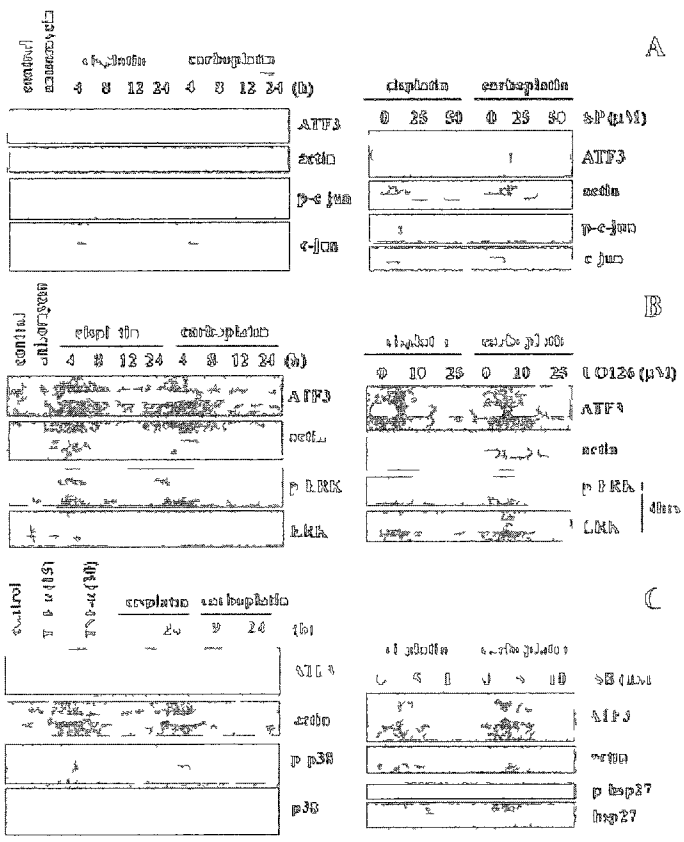
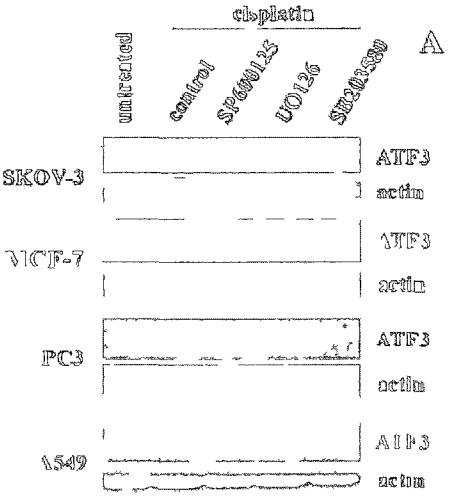

A
cisplatin
carboplatin
0 25 50 0 25 50 cP (μM)
ATF3
actin
p-c-jun
c-jun
B
cisplatin
carboplatin
0 10 25 0 10 25 U0126 (μM)
ATF3
actin
p ERK
ERK
C
cisplatin
carboplatin
0 5 10 0 5 10 SB (μM)
ATF3
actin
p hsp27
hsp27
control
cisplatin
carboplatin
4 8 12 24 4 8 12 24 (h)
ATF3
actin
p-c-jun
c-jun
p ERK
ERK
p38
p38

Figure 4


cisplatin
A
untreated
control
SP600125
UO126
SB203580
SKOV-3
ATF3
actin
MCF-7
ATF3
actin
PC3
ATF3
actin
A549
ATF3
actin

B

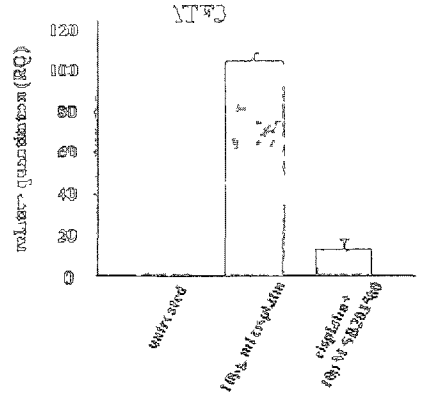

ATF3
relative quantification (RQ)
120
100
80
60
40
20
0
untreated
16h cisplatin
cisplatin + SB203580

Figure 4

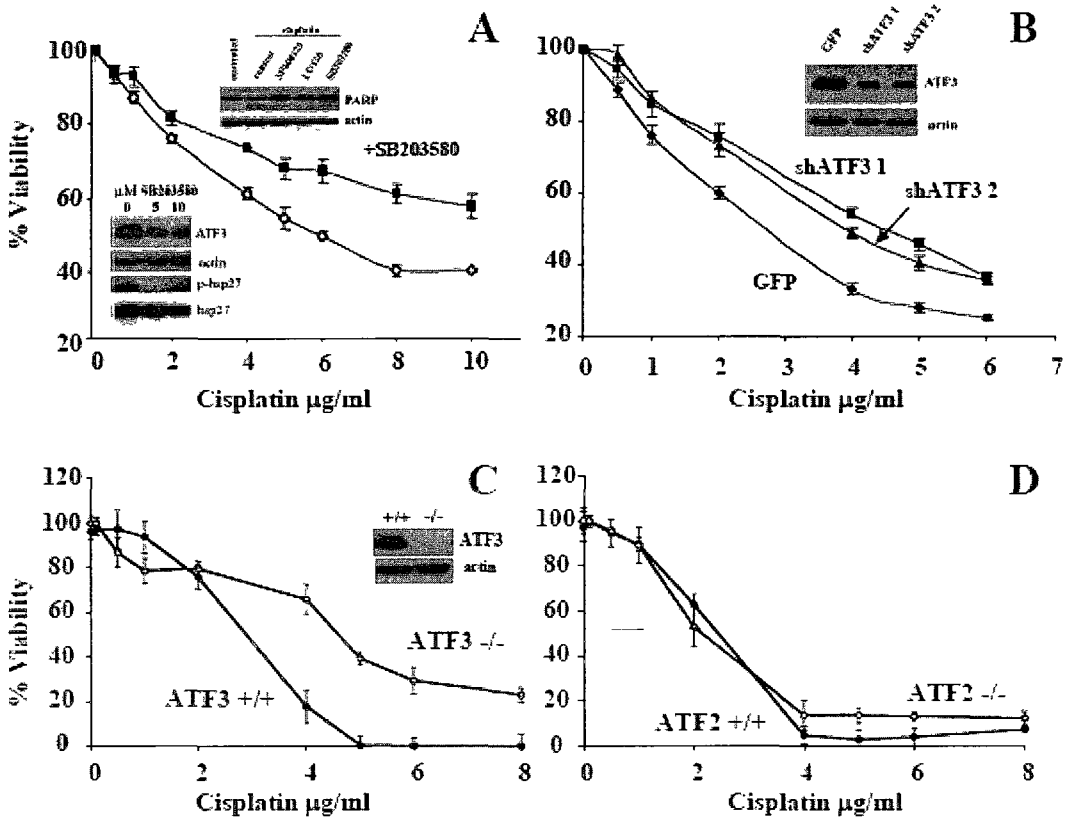

A
B
C
D
% Viability
Cisplatin µg/ml
+SB203580
PARP
actin
ATF3
shATF3 1
shATF3 2
GFP
ATF3 -/-
ATF3 +/+
ATF2 -/-
ATF2 +/+

Figure 6

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The DNA repair proteins BRCA1 and ERCC1 as predictive markers in sporadic ovarian cancer

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This study compares *Breast Cancer 1* (*BRCA1*) and excision repair cross complementation group 1 (*ERCC1*) expression as predictive markers and evaluates the *in vitro* enhancement of platinum sensitivity using targeted agents in sporadic ovarian cancer (OC). A retrospective study was performed of advanced stage OC patients receiving platinum-based chemotherapy. *BRCA1* and *ERCC1* mRNA expression was determined from frozen tissue of 51 patients. Median overall survival (OS) was longer for patients with lower *BRCA1* vs. higher *BRCA1* (46 vs. 33 months, $p = 0.03$). High *BRCA1* was predictive of poorer OS specifically in patients with residual disease (RD) <2 cm ($p = 0.03$). There was a non-significant association for patients with lower *ERCC1* and RD <2 cm in favor of improved OS and time to progression. Patients who expressed higher levels of both BRCA1 and ERCC1 mRNA had a shorter OS compared to patients with lower levels of either or both transcript (33 vs. 46 months, $p = 0.04$). When Cox proportional modeling was used by representing *BRCA1* and *ERCC1* mRNA expression as a continuous variable, both emerge as potential predictors of survival. OC cell lines were exposed to chemotherapy in combination with DNA repair pathway inhibitors and cell viability was assessed. *In vitro* histone deacetylase (HDAC) inhibition increased the sensitivity of A2780s cp cells to cisplatin and carboplatin but not to taxol, coincident with a significant decrease in BRCA1 and ERCC1 expression, suggesting that the compound directly targets DNA repair. In summary, this study shows that low *BRCA1* and *ERCC1* expression correlate with improved survival in advanced OC and HDAC inhibition induces synergistic cytotoxicity with platinum *in vitro*.

Key words: BRCA1; ERCC1; sporadic ovarian cancer; DNA repair; predictive marker

Epithelial ovarian cancer (OC) is generally diagnosed at an advanced stage resulting in a 5-year survival of only 20–40%. The standard treatment of OC is surgical debulking followed by platinum and taxane chemotherapy. Although 70% of patients with advanced disease initially respond to the first line regimen, early recurrences and platinum resistance are common obstacles in the management of this disease. Identifying reliable predictors of response and the use of novel therapeutic agents to enhance platinum efficacy are needed to improve the management of OC. Platinum resistance is multifactorial[1] and an important mechanism of resistance is increased tolerance to platinum DNA damage and enhanced repair of damaged DNA. In a variety of malignancies including OC, enhanced expression of the DNA repair proteins, *Breast Cancer 1* (BRCA1) and excision repair cross complementation group 1 (ERCC1) have correlated with resistance to platinum.[2–6] *In vitro* models have shown that targeting the expression of both BRCA1 and ERCC1 sensitizes cells to platinum-based chemotherapeutic agents suggesting a potential clinical application to help overcome platinum resistance in OC.

Mutations in the *BRCA1* tumor suppressor gene are associated with a significant risk of developing breast and OC, yet over 90% of OCs are believed to arise sporadically. While mutation of this gene is uncommon in sporadic disease, BRCA1 dysfunction, however, is frequently observed in OC.[9] Silencing of *BRCA1* through promoter methylation, decreased expression through gene deletion (loss of heterozygosity) or misregulation of related genes in the BRCA1 pathway is important in the pathogenesis of a significant proportion of sporadic tumors.[10,11] Recent clinical data suggests that tumor expression of BRCA1 has predictive value in patients without a *BRCA1* mutation.[2,9] A large correlative study on BRCA1 protein expression in 230 ovarian tumors with survival data on 152 patients revealed that patients with minimal BRCA1 expression had a longer overall survival (OS).[2] A recent study of 70 patients treated with platinum-based therapy concluded that low or intermediate levels of *BRCA1* mRNA were associated with improved survival compared to those with high levels. However, in this study, there were heterogeneous *ERCC1* levels in the high *BRCA1* group and a significant number of patients were treated with an outdated chemotherapy regimen, platinum and cyclophosphamide, which may not be reflective of patients receiving current chemotherapy. Although BRCA1 deficiency is generally associated with improved prognosis after platinum-based treatment, not all reports have validated this finding,[13] indicating that further studies are needed. Interestingly, low BRCA1 expression has been shown to have prognostic significance in sporadic breast cancer[1] and has correlated with prognosis after platinum-containing chemotherapy in non-small cell lung cancer (NSCLC),[1] suggesting BRCA1 may have a significant role as a biomarker in various tumor types.

In retrospective studies, elevated ERCC1 expression has correlated with platinum resistance in patients with colorectal,[15] gastric,[16] esophageal,[17] head and neck[1] and NSCLC.[19–21] Ovarian tumors from patients clinically resistant to platinum compounds have been shown to have elevated levels of ERCC1.[4,22] However, in a recent Gynecologic Oncology Group study of 170 cases of optimally resected stage III OCs, *ERCC1* mRNA expression and DNA adduct formation from peripheral blood leukocytes did not prove to be independent predictors of clinical outcome.[7] Unfortunately, tumor tissue was not available from study participants for direct correlation between tumor ERCC1 expression and patient outcomes. Therefore, the potential role of ERCC1 as a predictive marker of platinum sensitivity and as a therapeutic target requires further study in OC.

Abbreviations: BRCA1, Breast Cancer 1; [illegible] ERCC1, excision repair cross complementation group 1; [illegible] HDAC, histone deacetylase; [illegible] OC, ovarian cancer; OS, overall survival; RD, residual disease; [illegible] TTP, time to progression.

Grant sponsors: [illegible] The Ottawa Regional Cancer Foundation.

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The improved prognosis in BRCA1 deficient tumors is believed to be due to an increase in sensitivity to DNA damaging chemotherapeutic drugs such as platinum, as a result of the induction of irreparable DNA double strand breaks (DSB) through homologous recombination repair.[24] Targeting DNA repair mechanisms has thus been proposed as a therapeutic strategy to further enhance platinum sensitivity in BRCA1 deficient tumors. Rationale therapeutic targets that sensitize cells to platinum by inhibiting DNA DSB repair include inhibitors of the Poly(ADP ribose) polymerase 1 (PARP1) enzyme, an important component of the repair machinery[25] and cyclin dependent kinase 2 (CDK2) which induces growth arrest following DNA damage allowing time for the repair process to proceed before DNA replication can occur.[26] A novel group of agents that target histone deacetylases (HDAC) are also thought to enhance platinum based chemotherapeutics by loosening the chromatin structure and facilitating enhanced DNA targeting of these agents.[27] This class of inhibitors is currently being evaluated in Phase I and Phase II clinical trials, but their sensitivity in OC, particularly in combination with platinum, merits further study.

Thus, preliminary data suggests that the DNA repair proteins BRCA1 and ERCC1 are markers of platinum resistance, but their value as predictive markers in OC is not well established. Correlative studies in well defined patient populations are needed to proceed to a prospective study. We provide the first study that correlates tumor *ERCC1* expression with survival and compares *BRCA1* and *ERCC1* in combination as predictive markers in advanced OC patients treated with platinum based chemotherapy. We evaluate the efficacy of novel therapies on platinum sensitivity *in vitro* and study the effect of select molecular inhibitors on BRCA1 and ERCC1 in tumors of DNA repair pathways.

Material and methods

Patient samples and clinical data

The protocol was approved by the Ottawa Hospital Research Ethics Board. Samples were selected retrospectively based on the following criteria: advanced stage (stage III/IV), histological diagnosis of mesothelial or ovarian cancer predominantly serous histology, adjuvant platinum based chemotherapy and availability of at least 2 years of clinical follow up data. Flash frozen tumor was obtained from patients who gave informed consent to submit tissue to the Ovarian Cancer Tissue Bank at the Ottawa Health Research Institute between 1997 and 2005. All tumor tissue collected at surgery was confirmed by a pathologist before tumor was stored at −80°C.

Response rate and recurrences were evaluated according to RECIST criteria.[28] Complete response (CR) is defined as the disappearance of all clinical and radiological evidence of tumor, partial response (PR) is at least a 30% decrease in the size of a target lesion, progressive disease (PD) is either the appearance of new tumor lesions or at least a 20% increase in the size of the existing tumor and stable disease (SD) is neither sufficient decrease of tumor to qualify for a PR nor sufficient increase to qualify for PD. An elevation in serum CA 125 (Cancer Antigen 125) for assessment of recurrence was confirmed either by documented disease on clinical examination, measurable disease on CT or ultrasound. Time to progression (TTP) was determined from the time the patient completed chemotherapy to the time of documented disease recurrence. Overall survival (OS) was calculated from the date of surgery to the date of death.

RNA isolation and RT-PCR

Total RNA was extracted from 3 separate 30 mg samples of frozen tumor for each patient using the RNeasy® Kit (Qiagen, MD). RNA concentration was quantified using a NanoDrop MD-1000 spectrometer (Wilmington, DE). One microgram of total RNA was reverse transcribed to complementary DNA for quantitative real time reverse transcription polymerase chain reaction (RT-PCR) as previously described.[29] The Applied Biosystems ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification. A real time PCR reaction was carried out in a total volume of 25 µl and contained 2.5 µl of synthesized cDNA (42 ng), 1.25 µl of Taq Man Gene Expression Assay Primer/Probe (20X) (Applied Biosystems, BRCA1 Hs00173233_m1, ERCC1 Hs00157415), 12.5 µl of Taq Man Universal PCR Master Mix (2X) (Applied Biosystems, 4304437) and 8.75 µl of RNase free water for BRCA1 expression. The endogenous control for BRCA1 and ERCC1 was the housekeeping gene, human GAPDH (20X) (Applied Biosystems Hs4333764-F). The positive control for BRCA1 was MCF 7 (ATCC# HTB 22), and for ERCC1 the cisplatin sensitive ovarian cell line, A2780s.[30] Amplification conditions were 95°C for 5 min, 40 PCR cycles at 95°C for 15 sec and 60°C for 1 min. Three independent reactions from separate RNA extractions were used to determine the average gene expression and a standard deviation for each tumor. Replicate expression values resulting in a larger standard deviation were repeated using the original cDNA for the given sample and the 2 most consistent results were used for the final determination of average gene expression for each tumor.

Tissue culture

The A2780s and A2780cp cell lines (kindly provided by Dr. B. Vanderhyden, Ottawa Health Research Institute, Ottawa, ON) were maintained in Dulbecco's MEM Media Supplemented (Ottawa Regional Cancer Centre) supplemented with 10% fetal bovine serum (Medicorp, Montreal). Cells were exposed to cisplatin (carboplatin) and agents provided by the pharmacy at the Ottawa Hospital Regional Cancer Centre (Ottawa), alone and in combination with a PARP (3-Aminobenzamide), Cdk2, Cdk2 Inhibitor III) and HDAC inhibitor (MS-275), all from Calbiochem, diluted from a 10 mM stock in DMSO.

Western blotting

Protein samples were collected in RIPA buffer containing 1% Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, MO) and protein content was measured using a commercially available protein assay, BCA Protein Assay Kit, Pierce, Rockford, IL) and a Biomate3 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples were separated on 8–12% SDS polyacrylamide gel and transferred to a PVDF membrane (Immobilon P, Millipore, Billerica, MA). Blocking was carried out with 5% milk in Tris buffered saline with Tween 20 (TBS T). For all subsequent immunoblotting, antibodies were diluted in the appropriate concentration in 5% milk in TBS T. Blots were incubated with the following primary antibodies for 1 hour at room temperature or overnight at 4°C: mouse anti BRCA1 (1:200, D-9, Santa Cruz, Santa Cruz, CA), mouse anti-γ-H2AX (1:1000, JBW301, Upstate, Santa Cruz, CA), mouse anti ERCC1 (1:1000 D-10, Santa Cruz, Santa Cruz, CA), rabbit anti-cleaved Histone γ-H2AX (1:1000, Cell Signaling, Lake Placid, NY) and mouse anti-actin (1:5000, Sigma Aldrich, St. Louis, MO). Following 3 washes in TBS T, blots were incubated with the corresponding HRP labeled secondary antibody for 1 hour at room temperature. Visualization of protein bands was performed using the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and GeneSnap image acquisition system (Syngene, Frederick, MD).

MTT assay

In 96 well flat bottom microtiter plates (Nunc, Naperville, IL), approximately 5,000 cells/190 µl of cell suspension was used to seed each well. The cells were incubated overnight to allow for cell attachment and recovery. After treatment, 50 µl of 5 mg/ml solution in phosphate buffered saline of the MTT tetrazolium salt (Sigma) was added and incubated for an additional 6 hr at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 150 µl of 0.01 M HCl (10% SDS, Sigma) solution. The plates were then incubated [illegible]

TABLE I – SUMMARY OF PATIENT CLINICAL AND PATHOLOGIC DATA

	n = 51
Age at diagnosis	
31–40	5
41–50	7
51–60	18
61–70	14
71–80	5
>80	1
Stage	
II	6
III	41
IV	4
Tumor grade	
1	2
2	6
3	44
Histology	
Serous	39
Serous/endometrioid	9
Serous/clear cell	1
Mixed	1
Undifferentiated	1
Surgical procedure	
TAH/BSO + O	30
BSO/USO + O	9
TAH+/−BSO + [illegible]	10
TAH/BSO + O + PLND	3
Residual disease	
≤2 cm	—
>2 cm	29
First line chemotherapy response	
CR	29
PR	17
SD	1
PD	4
First line chemotherapy regimen	
Carboplatin/taxol	26
Carboplatin	1
Cisplatin/taxol	18
Cisplatin/cyclophosphamide	1
Carboplatin/taxol/epirubicin	3
Cisplatin/topo, carboy/taxol	2

TAH, total abdominal hysterectomy; BSO/USO, bilateral/unilateral salpingooophorectomy; O, omentectomy; PLND, bilateral pelvic lymph node dissection; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

MRX Microplate Reader from Dynex Technologies at 570 nm to determine the optical density of the samples.

Statistical analyses

In the description of subjects, categorical data were expressed by percentages and continuous variables as the mean and standard deviation. mRNA expression by RT-PCR was modeled as a continuous variable in the primary analysis, with *BRCA1* and *ERCC1* levels log-transformed to achieve normality. Cox Proportional Hazards models were used to determine the relationship between OS and TTP across *BRCA1* and *ERCC1* levels. The hazard ratio (HR) and 95% confidence interval (95% CI) were reported in separate univariate models for both *BRCA1* and *ERCC1*. Patient age, stage, performance status and residual disease post surgery are independent predictors of overall survival in OC.[11] Among these, only performance status was not available for analysis in this study. Each of the remaining risk factors was assessed by separate univariate Cox models and those found to be significant were adjusted for in a multivariate Cox analysis.

In order to compare with other published studies, mRNA expression was expressed as a dichotomous variable, dividing all patients into 2 groups, with relative higher and lower mRNA expression. The Kaplan Meier method was used to compare overall survival and time to progression curves for *BRCA1* and *ERCC1* levels divided into 2 groups using their respective median as the cutoff point. The log-rank test was used to assess the effect between groups with an alpha level of 0.05 used as the cutoff for statistical significance. Kaplan Meier curves were performed in the entire sample as well as in patients stratified according to residual disease status (≤2 cm and >2 cm). Correlation analysis was performed between *BRCA1* and *ERCC1*. All analyses were performed using SAS version 9, Cary, NC.

TABLE II – COX PROPORTIONAL HAZARD MODEL FOR *BRCA1* AND *ERCC1* (n = 51)

	hazard ratio	95% CI	p
Overall survival			
Log BRCA1	1.26	0.92, 1.73	0.15
Log BRCA1[1]	1.09	0.78, 1.52	0.60
Log ERCC1	1.56	1.00, 2.45	0.05
Log ERCC1[1]	1.45	0.91, 2.32	0.12
Time to progression			
Log BRCA1	1.10	0.85, 1.43	0.47
Log BRCA1[1]	1.00	0.76, 1.31	0.99
Log ERCC1	1.25	0.82, 1.90	0.30
Log ERCC1[1]	1.10	0.77, 1.84	0.44

[1]Adjusted hazard ratios for residual disease status.

Results

Patients

Fifty one patients diagnosed with OC between 1997 and 2005 underwent surgical staging and adjuvant chemotherapy at The Ottawa Hospital (Table I). The follow up period was a minimum of 2 years for deaths and up to 101 months. The mean patient age was 57.1 ± 11.5 years (range, 31–81). One patient was lost to follow up after 1 year. Consent was not obtained for genetic testing in this cohort and therefore, germline mutations for BRCA1 were unknown at the time of patient selection. The patient cohort is composed mainly of patients with high grade advanced stage, papillary serous tumors, the most common presentation of OC at diagnosis. Twenty nine (56%) patients had RD >2 cm after surgery, several of whom presented with significant bulky RD (> 10 cm), and 22 patients (44%) had RD ≤ 2 cm. All patients had platinum based chemotherapy in the first line setting including 5 patients who participated in clinical trials (OV 16, 2; OV 14, 3). Only 2 of the 51 women (4%) did not receive carboplatin or cisplatin plus taxol as the basis of the first line regimen. Ninety per cent of patients had either a CR or PR after surgery and cytotoxic chemotherapy.

The evaluation of BRCA1 and ERCC1 as predictive markers in ovarian cancer

There were too few patients with young age, grade 1 tumors and stage II disease to factor in the multivariate analysis. RD status was a significant confounder and was accounted for in the adjusted Cox proportional model (Table II). The unadjusted hazard ratios for *BRCA1* and *ERCC1* for overall survival are 1.26 [0.92, 1.73], and 1.56 [1.00, 2.45], respectively. Although this effect was diminished when adjusted for RD status, the trend in this relatively small cohort was in favor of decreased survival with higher expression of both *BRCA1* and *ERCC1*, all HR > 1. There was also a non-significant association between a poorer TTP with increasing *ERCC1*.

BRCA1 mRNA expression for each tumor was compared to the human breast adenocarcinoma cell line MCF7, the positive control, which is known to express significant levels of BRCA1. MCF7 has been used extensively as a comparative sample in many studies, pertaining to BRCA1 and was assigned an expression value of 1.0. When patients were divided according to relatively higher and lower mRNA expression with respect to MCF7, the 2 groups were similar with respect to age, stage, grade and RD status. There was no statistically significant difference in the propor-

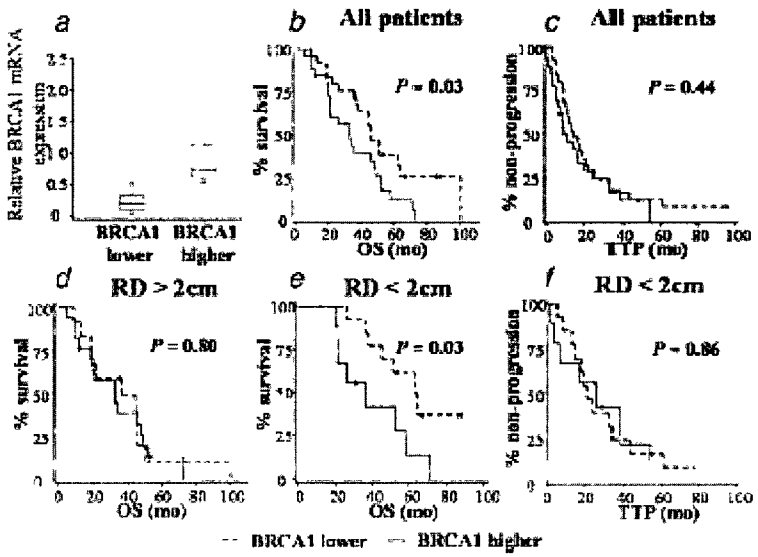

FIGURE 1 *BRCA1* mRNA levels expressed as a dichotomous variable comparing 51 patients divided in BRCA1 higher (>0.54) and BRCA1 lower (<0.54) groups. (*a*), Median mRNA levels, 25th and 75th percentiles, and highest and lowest values shown for each group. (*b* and *c*), OS and TTP for all patients divided into lower and higher groups. (*d* and *f*), *BRCA1* higher and *BRCA1* lower groups compared in terms of OS and TTP for patients separated according to RD > 2 cm (*e* = 24) and RD < 2 cm (*e* = 22).

tion of responders (CR and PR) between the higher and lower groups for *BRCA1* ($p = 0.28$) and *ERCC1* ($p = 0.09$). The mean age at diagnosis for patients in *BRCA1* lower was 56.9 ± 12.0, [31–81] and *BRCA1* higher was 57.3 ± 11.2, [33–80]. Tumors in the *BRCA1* lower group had relative mRNA expression levels <0.52, and in the *BRCA1* higher group had mRNA levels >0.53 (Fig. 1). Only 9 patients were still alive at the end of the study, and 7 of 9 of these patients were in the *BRCA1* lower group. Kaplan Meier estimates of survival for all patients indicate the median OS for the *BRCA1* lower group was 46 months, 95% CI [37–64], and for the *BRCA1* higher group was 33 months, 95% CI [21–48], $p = 0.03$, suggesting that patients with relatively lower *BRCA1* expression showed an increased likelihood of survival. When patients were stratified according to RD status, higher values of *BRCA1* were predictive of a poorer OS in those patients only with RD < 2 cm ($p = 0.03$), but not in the group of patients with RD >2 cm ($p = 0.80$). At the end of the follow up period, 46 of 51 patients had recurrent disease and 5 of 51 patients had not yet experienced a recurrence. There was no statistically significant difference in median TTP between *BRCA1* lower and *BRCA1* higher groups, regardless of RD status.

The range of relative mRNA expression for *ERCC1* was greater than that for *BRCA1*. Tumors in the *ERCC1* lower group had mRNA expression <0.86 and in the *ERCC1* higher group had mRNA levels >0.91, relative to the positive control A2780s (Fig. 2). The mean age at diagnosis in *ERCC1* lower was 56.8 ± 11.8, [31–81] and for *ERCC1* higher was 57.4 ± 11.4, [33–80]. For all patients, the median OS in *ERCC1* higher was 36 months, compared to 46 months in the *ERCC1* lower group ($p = 0.14$). Similar to *BRCA1*, median survival and TTP in patients with RD < 2 cm were longer in the *ERCC1* lower group, although this did not reach statistical significance (OS $p = 0.10$ and TTP $p = 0.11$). The mRNA expression levels of *ERCC1* correlate with those of *BRCA1* ($p = 0.04$), and tumors with both *BRCA1* higher and *ERCC1* higher were compared to groups where either *BRCA1* or *ERCC1* were in the lower group (Figs. 4a and 4b). Those patients who expressed higher levels of both mRNA transcripts had a median OS of 33 months, compared to 46 months for those patients that did not ($p = 0.04$).

The in vitro effect of drug sensitivity on BRCA1 and ERCC1 expression in OC

When 3 molecular inhibitors were combined with standard chemotherapeutic agents in A2780s and A2780cp cells, there was no improvement in drug sensitivity when the PARP inhibitor and CDk2 inhibitor were added to chemotherapy drugs (Fig. 3). Only the HDAC inhibitor significantly increased sensitivity of A2780s and A2780cp cells to cisplatin and carboplatin cytotoxicity. However, there was no effect of this inhibitor on the cytotoxicity of taxol, which is not a DNA damaging agent. Both OC cells lines, A2780s and its cisplatin resistant derived clone A2780cp, expressed relatively high levels of *BRCA1* and *ERCC1* mRNA (Fig. 4c). Upon exposure of cell lines to cisplatin, carboplatin and taxol, there were subtle changes in *BRCA1* and *ERCC1* mRNA levels, but no significant differences in protein expression (Figs. 4a and 4c). Treatment with the PARP and CDk2 inhibitors had relatively no effect on BRCA1 and ERCC1 mRNA transcripts and protein levels. However, in both the A2780s and A2780cp, there was a significant decrease in mRNA and protein levels of both *BRCA1* and *ERCC1* with the HDAC inhibitor, suggesting that this compound directly targets DNA repair by modulating the expression of key regulators of this pathway.

Discussion

In this retrospective study of a homogeneous group of 51 advanced OC patients who were treated with adjuvant platinum and paclitaxel, we have correlated low levels of *BRCA1* and *ERCC1* mRNA expression with a better OS and this effect was

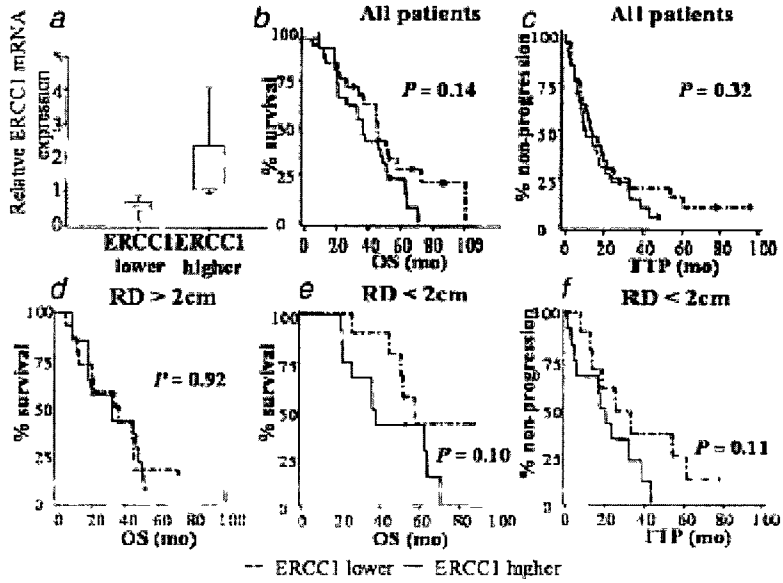

FIGURE 2. *ERCC1* mRNA levels expressed as a dichotomous variable comparing 51 patients divided in *ERCC1* higher (>0.91) and *ERCC1* lower (≤0.91) group (a). Median mRNA levels, 25th and 75th percentiles, and highest and lowest values shown for each group (*b* and *c*). OS and TTP for all patients divided into upper and lower groups (*d* and *f*). *ERCC1* higher and lower group compared in terms of OS and TTP for patients separated according to RD > 2 cm and RD ≤ 2 cm.

more marked in patients who have had an optimal surgical resection to ≤2 cm. Consistent with other reports, our study supports *BRCA1* as a predictive biomarker in OC when mRNA expression is represented as a dichotomous variable. Importantly, we related *BRCA1* expression to RD status and demonstrate that in the presence of RD > 2 cm, the predictive value of *BRCA1* is lost. This data suggests that significant RD is an overriding prognostic factor that should be accounted for in future biomarkers studies in OC. When Cox proportional modeling is used by representing mRNA as a continuous variable, both *BRCA1* and *ERCC1* expression emerge as potential predictors of survival, but a larger study is need to validate this effect. In addition, we have demonstrated synergistic cytotoxicity with the addition of an HDAC inhibitor to cisplatin and carboplatin in A2780s and A2780cp OC cells, whereby the mechanism of action involves decreased expression of both DNA repair proteins, BRCA1 and ERCC1.

Currently, there are no clinically relevant biomarkers which predict for chemosensitivity and overall prognosis in OC. The serum glycoprotein, CA 125, is the only tumor marker that is used clinically to follow response to chemotherapy.[37] However, baseline CA 125 does not correlate with platinum resistance, overall prognosis, or the likelihood of optimal tumor resection.[38] Despite the heterogeneity with respect to the clinical and pathologic presentation in OC, most patients who receive first line chemotherapy are treated uniformly with platinum and paclitaxel. Thus, there is a need to identify molecular markers which may impact on therapeutic options based on tumor biology. Reliable predictors of platinum resistance may identify a subgroup of patients that is unlikely to respond to platinum-based treatment, decrease toxicity from unnecessary chemotherapy and assist clinicians in directing patients to participation in clinical trials.

This study is in agreement with preclinical data which suggests that tumor expression of BRCA1 may have predictive value in patients with no *BRCA1* mutation. Resistant variants to cisplatin of the breast and ovarian cell lines MCF 7/R and SKOV 3/R resulted in increased sensitivity to cisplatin with transient inhibition of *BRCA1*.[39] *BRCA1* deficient mouse ovarian epithelial cells were also shown to have increased sensitivity to platinum.[40,41] Furthermore, it has been shown in breast cancer that BRCA1 functions as a differential modulator of chemotherapy induced apoptosis in the presence of a range of cytotoxic agents.[5] Overexpression of *BRCA1* in OC cells in vitro led to resistance in DNA damaging agents including cisplatin, etoposide and doxorubicin.[38] *BRCA1* has been implicated in the spindle assembly checkpoint, such that down regulation of *BRCA1* causes disruption of the mitotic spindle.[39–41] This may account for resistance to the microtubule-interfering agent paclitaxel in cells with inhibited endogenous *BRCA1* expression.[39,42] Therefore, preclinical data implies that patients with low levels of BRCA1 may benefit from single agent chemotherapy with a platinum drug, whereas those with high levels may benefit from a single agent taxane. Since all but 1 patient in this study received combination platinum and paclitaxel, one must consider that in the clinical setting, the optimal benefit of platinum may not be attained due to a possible negative effect of taxanes in BRCA1 deficient tumors. Although Isaacs's group has shown that reduced *BRCA1* expression confers increased resistance to taxanes in vitro, there was a non significant trend in their retrospective patient analysis to suggest that patients with high tumor levels of *BRCA1* may gain a survival advantage with taxane based chemotherapy.[5]

In this patient cohort, Cox proportional modeling revealed a significant correlation of low *ERCC1* expression with a better OS, implying that *ERCC1* may also be a relevant predictive marker in OC. Our study supports preclinical data demonstrating that inhibition of the ERCC1 protein by antisense RNA expression in A2780 and the cisplatin resistant OC cell line OVCAR10, enhanced platinum sensitivity in vitro.[43] This data is also in agreement with a gene analysis of tumor from 15 OC patients, clinically resistant to platinum which showed higher mRNA levels of *ERCC1* when compared to tumor from 15 patients with sensitive disease. In

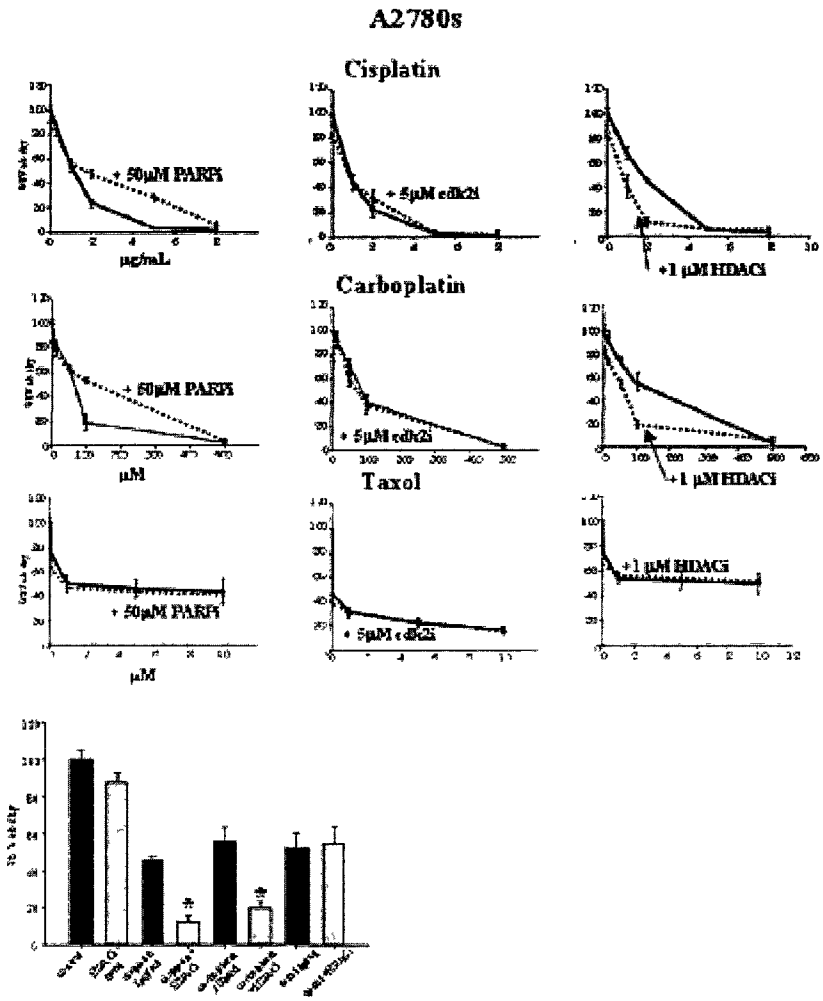

FIGURE 3 MTT viability assays comparing the responses of 0–8 ng/ml cisplatin, 0–500 μM carboplatin or 0–10 μM taxol alone or with the coadministration of 50 μM PARPi, 5 μM cdk2i or 1 μM HDACi. Cell viability was assayed with the activity of untreated cells taken to be 100%. *Combination of treatments that displayed significant differences in MTT activity compared to either agent alone ($p < 0.01$, Paired T test). MTT+ were analyzed at 48 h consistent with the method used in the National Cancer Institute.[5]

NSCLC among patients treated with platinum based chemotherapy. low levels of ERCC1 has been associated with an improved overall survival[19,20] but this effect was not consistently shown.[45,46] In the only prospective randomized clinical trial testing customized chemotherapy in advanced NSCLC, ERCC1 mRNA predicted for response to cisplatin based chemotherapy in 346 patients.[47] Some researchers have demonstrated that in early stage NSCLC surgically resected for cure, ERCC1 expression was also prognostic in favor of longer survival.[48–50] However the finding was not confirmed in all studies.[51] Overall, the effect of ERCC1 as a biomarker of prognosis is not yet conclusive in lung cancer and there is comparatively little data available in OC. ERCC1 plays a rate limiting role in excising DNA on the 5′ side of a lesion, such as a platinum induced DNA adduct, and cells without functional ERCC1 do not undergo DNA adduct repair. The nucleotide excision repair (NER) pathway is the main mechanism of repairing cisplatin induced DNA cross links and unblocking transcription and thus is highly relevant to the study of DNA repair mediated chemoresistance in OC. While ERCC1 is crucial to NER, evidence also supports its role in homologous recombination repair as ERCC1 was shown to be involved in the processing of interstrand cross link induced DSB.[41] Thus, it is an intriguing possibility that BRCA1 and ERCC1 may be linked through a common DNA repair function, such as DSB repair.

The current study suggests that *BRCA1* and *ERCC1* levels correlate with each other, which is important in the consideration of

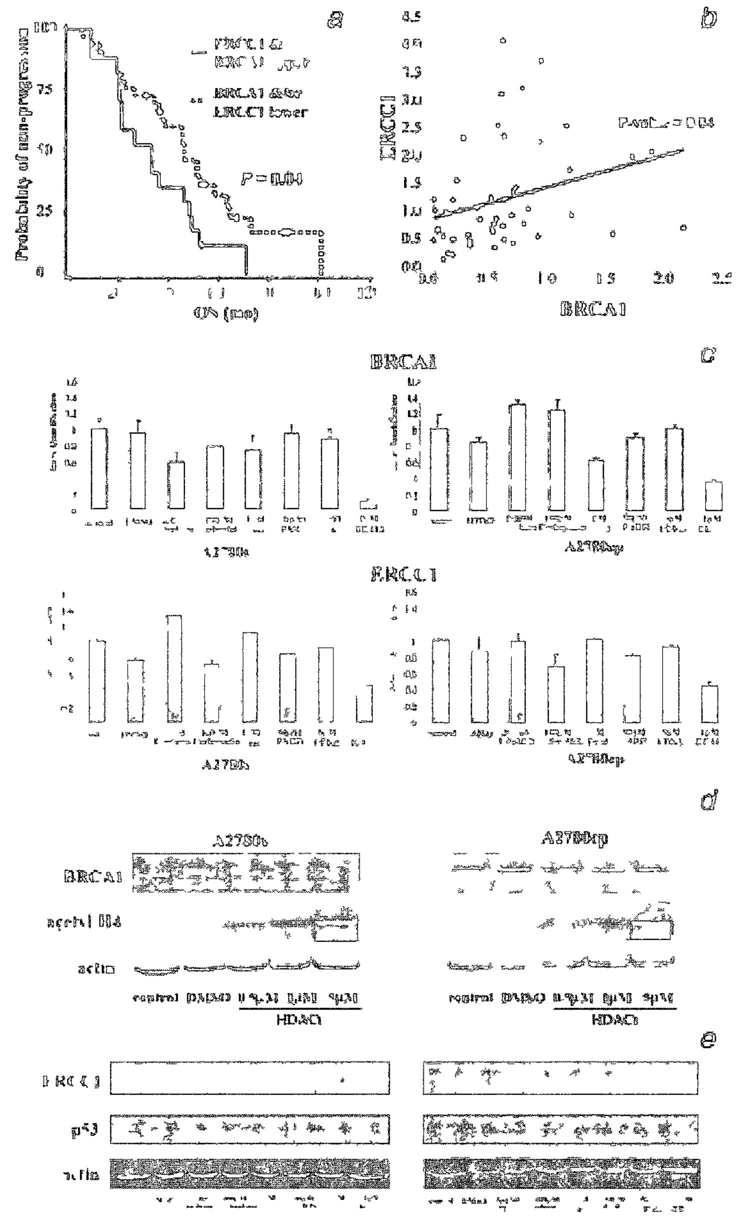

FIGURE 4. (*a*) High levels of both BRCA1 and ERCC1 mRNA in patient tumors predicts for a poorer outcome compared to when either or both are low. (*b*) BRCA1 and ERCC1 mRNA levels correlate with each other: ERCC1 = 0.5[illegible] BRCA1 + 0.[illegible]. (*c*) Levels of BRCA1 and ERCC1 mRNA analyzed by real time quantitative RT-PCR following 24 hr treatment as indicated. Fold changes were calculated following normalization to *gapdh* levels (ΔΔCt) and expressed as mean (+ SEM). (*d*) BRCA1, acetyl H4 and actin protein following treatment with control (untreated), DMSO and HDACi in A2780s and A2780cp as indicated. (*e*) Western blot analysis of ERCC1 and p53 in A2780s and A2780cp cells after treatment with HDACi, CDK2i, PARPi and relevant chemotherapeutics.

future studies. Firstly, the combination of markers may have greater predictive value than one single marker. We have shown that high levels of both *BRCA1* and *ERCC1* mRNA are associated with a poorer survival compared to low levels of either marker, and this important observation requires further study. Secondly, even with numerous studies suggesting the value of BRCA1 as a predictive biomarker in various malignancies, expression levels of a subset of DNA repair pathway constituents may provide a more robust molecular profile of chemoresponsiveness and prognosis. The targeted expression of the SV40 T/t antigen led to the discovery of a conserved gene transcriptional signature in multiple epithelial tumors.[52] This genetic signature was found to be activated uniquely in tumors with aberrant p53, Rb or BRCA1 expression, and in breast, prostate and lung cancer, associated with an aggressive phenotype and poor prognosis, suggesting that they may represent rational candidates for the preclinical testing of biologically important targets.

DNA repair pathways are frequently defective in malignant cells and targeting such defects seems to be a more rational therapeutic approach than direct gene therapy.[53,54] HDAC inhibitors are a promising new class of anticancer therapeutics which are have been tested in phase I and phase II clinical trials.[55–57] The interaction between histone acetyl transferases and histone deacetylase enzymes regulates gene expression by modifying chromatin transcription in various malignancies, and BRCA1 is known to play a role in altering chromatin structure. The mechanism by which HDAC inhibition is thought to enhance platinum induced cytotoxicity is through chromatin relaxation allowing for increased DNA damage in transcriptionally active DNA regions.[27] Our group has demonstrated that in addition to this mechanism, HDAC inhibitors also sensitize OC cells to platinum by decreasing the expression of both BRCA1 and ERCC1. This represents a novel mechanism of activity of this class of agents that requires further study. Histone H2AX, a well known characterized marker of DNA double strand breaks, is known to bind to the BRCA1 carboxyl terminal (BRCT) domain of the BRCA1 gene.[59] It has been suggested that HDAC inhibitors effectively hinder tumor growth when combined with DNA damaging agents, such as ionizing radiation and DNA damaging chemotherapeutics, by the inhibition of DNA double strand break repair through prolonged phosphorylation of H2AX. The HDAC inhibitor PCI 24781, which inhibits various HDAC isoforms, led to a significant reduction in gene transcription associated with homologous recombination repair including *RAD51*.[60] Interestingly, this compound resulted in a synergistic effect on apoptosis in combination with a PARP inhibitor, suggesting that this drug combination may be particularly effective in tumors with defects in homologous recombination repair. PARP1 is a rationale therapeutic target in *BRCA1* deficient cancer cells since BRCA1 dysfunction sensitizes cells to inhibition of this enzyme.[61,62] In an IHC study of PARP expression in a cohort of ovarian serous carcinomas, strong PARP expression was determined in 76% of cases and this group correlated with a poorer outcome compared to patients with low expression.[63] In the present study, both A2780s and A2780cp cell lines exhibited relatively high levels of *BRCA1* and *ERCC1* mRNA. Therefore, it is not unexpected that treatment with a PARP inhibitor did not demonstrate *in vitro* cytotoxicity.

There are several limitations to this retrospective analysis that merit consideration. Our study is unable to address whether *BRCA1* and *ERCC1* mRNA levels are prognostic, that is, the ability of these markers to determine clinical outcome independent of treatment given.[64] The ability to perform a study in stage I OC patients to assess the prognostic value of a tumor marker is challenging due to the limited number of early-stage OC patients who do not receive chemotherapy. One must also consider that in the current study, mutational status for *BRCA1* was unknown at the time of patient inclusion into the analysis. While this information is valuable, since a *BRCA1* mutation may in itself lend to a more favorable outcome, we propose that as a predictive marker, *BRCA1* expression is considered as a spectrum from low to high, regardless of the mechanism of *BRCA1* inactivation, whether it is via gene mutation, gene deletion or an epigenetic mechanism. Furthermore, we acknowledge that in a subset of patient tumors in the study, there was intrapatient variability between replicate gene expression levels. In such cases, outlier values were repeated using the original cDNA in order to obtain the most consistent triplicate values for each sample. In future studies, microdissection would likely ensure better accuracy of representative tumor RNA and obviate the potential problem incorporating normal tissue in the interpretation of results.

The present study provides additional evidence to support the tumor mRNA levels of both *BRCA1* and *ERCC1*, and importantly their combination, as having value as predictive markers in OC. The data from this and other studies indicate the need to further evaluate these markers in a prospective trial. Furthermore, this is the first study which demonstrates that HDAC inhibitors effectively target the expression of both *ERCC1* and *BRCA1* in OC cells and enhance the cytotoxic effects of cisplatin and carboplatin but not taxol, suggesting a novel therapeutic mechanism of activity of these compounds that may be exploited therapeutically.

Acknowledgements

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EDUCATION

2008 to Present	Masters of Science, Biochemistry - Human and Molecular Genetics University of Ottawa, Ottawa, ON
2007	Honours, Bachelor of Science, Biochemistry, Co-operative Education University of Guelph, Guelph, ON - Entrance Scholarship
2002	Ontario Secondary School Diploma Mother Teresa Catholic High School, Nepean, ON - Ontario Scholar

EMPLOYMENT HISTORY

May 2007 – April 2008 **Special Student**
Supervisor: Dr. Jim Dimitroulakos
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September – December 2005 **Coop Student**
Hazardous Materials Information Review Commission of Canada,
Health Canada, Ottawa, ON

January – April 2005 **Coop Research Assistant**
Supervisor: Dr. John Dawson
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January – April 2004 **Scientific Evaluator/Research Assistant**,
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Toxicological Evaluation Section, Natural Products and Food Branch,
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Health Canada, Ottawa, ON

PUBLICATIONS

2009 Weberpals J, **Garbuio K**, O'Brien A, Clark-Knowles K, Dimitroulakos J. Histone deacetylase inhibition targets BRCA1 expression: Potential novel therapeutic implications. *Molecular Cancer Therapeutics*. Submission pending.

2009 St. Germain C, Niknejad N, Ma L, **Garbuio K**, Hai T, Dimitroulakos J. Cisplatin induces cytotoxicity via the mitogen-activated protein kinase pathways and Activating Transcription Factor 3. Accepted to *Neoplasia* December 2009.

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

ABSTRACTS/POSTERS/PRESENTATIONS

November 2009 Weberpals J, **Garbuio K**, O'Brien A, Clark-Knowles K, Dimitroulakos J Histone deacetylase inhibition targets BRCA1 expression Potential novel therapeutic implications (Abstract and Poster) Submitted to the American Association for Cancer Research (AACR) – National Cancer Institute (NCI) - European Organization for Research and Treatment of Cancer (EORTC) International Conference on Molecular Targets and Cancer Therapeutics Boston, MA

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

June/July 2008 Weberpals J, **Garbuio K**, O'Brien A, Clark-Knowles K, Doucette S, Antoniouk O, Goss G, Dimitroulakos J The DNA repair proteins *BRCA1* and *ERCC1* as predictive markers in sporadic ovarian cancer (Abstract and Poster) Submitted to the 2nd International Conference on Ovarian Cancer Rhodes, Greece

May/June 2008 Weberpals J, Clark-Knowles K, O'Brien A, Antoniouk O, **Garbuio K**, Haidari L, Dimitroulakos J *BRCA1* and *ERCC1* expression as predictors of platinum response in ovarian cancer (Abstract, Poster, and Presentation) Submitted to the 44th American Society of Clinical Oncology Annual Meeting Chicago, Illinois

May 2008 Weberpals J, **Garbuio K**, O'Brien A, Clark-Knowles K, Doucette S, Antoniouk O, Goss G, Dimitroulakos J The DNA repair proteins *BRCA1* and *ERCC1* as predictive markers in sporadic ovarian cancer (Abstract and Presentation) Submitted to the 4th Canadian Conference on Ovarian Cancer Research Montreal, Quebec

May 2008 **Garbuio K**, O'Brien A, Weberpals J, Dimitroulakos J Identification of ATF3 as a potential novel therapeutic target of ovarian carcinomas (Abstract and Poster) Submitted to the 4th Canadian Conference on Ovarian Cancer Research Montreal, Quebec

October 2007 Weberpals J, Clark-Knowles K, Antoniouk O, Haidari L, **Garbuio K**, Dimitroulakos J Low *BRCA1* and *ERCC1* expression correlates with improved survival in ovarian cancer (Abstract and Poster) Submitted to the American Association for Cancer Research (AACR) – National Cancer Institute (NCI) - European Organization for Research and Treatment of Cancer (EORTC) International Conference on Molecular Targets and Cancer Therapeutics San Francisco, CA

June 2007 Weberpals J, Clark-Knowles K, Haidari L, Crane C, Antoniouk O, **Garbuio K**, Dimitroulakos J Targeting the *BRCA1*-dependent DNA damage and repair pathway in sporadic epithelial ovarian cancer (Abstract and Poster – Award for Best Poster) Submitted to the 28th Annual General Meeting of the Society of Gynaecologic Oncologists of Canada Ottawa, ON

LEADERSHIP and VOLUNTEERING

2009 Acted as a **Supervisor-Mentor** for a high school student participating in the **Sanofi-Aventis BioTest Challenge**, the Eastern Ontario arm of the Canada-Wide Science Fair Ashley Millette received the Medical Laboratory Association award, the University of Ontario Innovation award, and an admission scholarship to the University of Ottawa for her project investigating the chemotherapeutic potential of ommochromes as compared to Pteridines Her awards also included first place, best high school project, and best in fair all around

2006 Donated time to an **Inpatient Medical Unit at the Queensway Carleton Hospital**, Nepean, ON, acting as a **volunteer** to bring ice water to patients and offer friendly visits ensuring a more tolerable stay

2006 Acted as **a medical volunteer** at the **Athletic Injury Clinic for the Ontario Summer Games** (August 9th to 13th Ottawa, ON), working to manage the office, distribute first aid kits to the various sport venues, and direct phone calls concerning injured athletes for the appropriate treatment