Enhancement of Cisplatin Cytotoxicity by Disulfiram Involves Activating Transcription Factor 3

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Abstract. *Background: Activating transcription factor 3 (ATF3), a stress-inducible gene, is a regulator of cisplatin-induced cytotoxicity, and enhancement of the ATF3 expression potentiates this cytotoxicity. Materials and Methods: ATF3 expression and its binding to the transcription target CHOP were evaluated by western blot and chromatin immunoprecipitation (ChIP), respectively, in a panel of five cell lines (WI38, MCF7, PC3, A549). MTT assays were employed to assess the effects of many drugs, including disulfiram, on cell viability. Results: ATF3 protein expression was up-regulated after cytotoxic doses of cisplatin treatment and it directly bound to the CHOP gene promoter, increasing this pro-apoptotic protein's expression. In a library screen of 1200 compounds, disulfiram, a dithiocarbamate drug, was identified as an enhancer of the cytotoxic effects of cisplatin. This increased cytotoxic action was synergistic and likely due to their ability to induce ATF3 independently. Conclusion: Understanding the role of ATF3 in cisplatin-induced cytotoxicity will lead to novel therapeutic approaches that could improve this drug's efficacy.*

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

Cisplatin covalently binds DNA to form bulky adducts that block replication and transcription, which leads to G_2 phase cell cycle arrest (9). It is the recognition of DNA damage by a host of proteins which activate pro-survival and pro-apoptotic signals that determine the cell's fate (10). Cisplatin has pleiotropic effects on the cell and investigating cellular pathways of cisplatin cytotoxicity may lead to the development of novel targeted therapies. For example, the mitogen-activated protein kinase (MAPK) cascade is an extracellular stress response pathway that includes three kinase members: extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal protein kinase (JNK) [also known as stress-activated protein kinase (SAPK)], and p38, all of which are activated by cisplatin (10). This pathway is important for the cytotoxic action of cisplatin since inhibition of any member can attenuate cisplatin-induced apoptosis (2, 11).

Activating transcription factor (ATF) 3 is a member of the ATF/cAMP-responsive element binding (ATF/CREB) family of basic region-leucine zipper (bZip) transcription factors (12). There are several transcription factor binding sites located within the *ATF3* gene promoter (13, 14). The expression of *ATF3* mRNA is transient due to its ability to repress its own promoter (15). Evidence suggests that ATF3 may repress or activate the transcription of target genes depending on its dimerizing partner and the promoter context

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(16). ATF3 plays a central role downstream of the integrated stress response (ISR) pathway that is activated upon viral infection, oxidative stress, endoplasmic reticulum (ER) stress and nutritional stress (17).

Identifying the mechanisms regulating cisplatin-induced apoptosis will lead to the discovery of novel therapeutic approaches. *ATF3* induction by cisplatin was recently shown to act though the MAPK pathway, the suppression of which is implicated in cisplatin resistance (18, 19). Therefore, combining cisplatin treatment with an inducer of *ATF3* has the potential to sensitize cells to its cytotoxic effects. Increased cisplatin cytotoxicity was demonstrated with the histone deacetylase (HDAC) inhibitor M344, in which the combination induces the ISR and the co-operative cytotoxicity was dependent on *ATF3* expression (20). Inhibitors of HDAC are relatively non-specific and can affect the expression of thousands of genes, including *ATF3*. Identifying more specific *ATF3* inducers that can be readily evaluated clinically may represent a potential novel combination therapeutic approach. In this study, we identified disulfiram in a chemical library screen as an enhancer of cisplatin-induced cytotoxicity. We demonstrate that this combination induces synergistic cytotoxicity and *ATF3* expression was enhanced by their combination.

Materials and Methods

Tissue culture. The WI38 (normal lung fibroblast), MCF7 (breast adenocarcinoma), PC3 (prostate adenocarcinoma), and A549 (NSCLC) cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line SKOV3 (ovarian adenocarcinoma), was kindly provided by Dr. Barbara Vanderhyden (Ottawa Hospital Research Institute, ON, Canada). All cell lines were maintained in Dulbecco's-modified eagle's medium (D-MEM) (Media Services, Ottawa Hospital Regional Cancer Centre, Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (Wisent Inc.; St.Bruno, QC, Canada) and 100 µg/ml penicillin-streptomycin (Invitrogen; Carlsbad, CA, USA). Unless otherwise described, cells were treated with cisplatin (provided by the pharmacy at the Ottawa Hospital Regional Cancer Centre) alone or in combination with a 24 h pre-treatment of disulfiram [10 mM stock diluted in dimethyl sulfoxide (DMSO), Sigma-Aldrich; St.Louis, MO, USA].

2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In a 96-well flat-bottomed plate (Corning Costar #3595; Corning, NY, USA) 4,500 cells/150 µl of cell suspension were used to seed each well. The cells were incubated overnight at 37°C to allow for cell attachment and recovery. Cells were treated with cisplatin alone and incubated for 48 h at 37˚C, or were treated with disulfiram for 24 h followed by treatment with both disulfiram and cisplatin for 24 h. Following treatment, 42 µl of a 5 mg/ml solution of the MTT tetrazolium substrate (Sigma-Aldrich) in phosphate buffered saline (PBS) were added to each well and incubated for 4 h at 37˚C. The resulting violet formazan precipitate was solubilised by the addition of 82 µl of a 0.01 mol/l HCl/10% sodium dodecylsulphate (SDS) (Sigma-Aldrich) solution, and plates were incubated further at 37˚C for 24-72 h. The plates were analyzed on a Synergy Mx Monochomator-Based Multi-Mode Microplate Reader using the Gen5 software, both from Biotek Instruments (Winooski, VT, USA), at 570 nm to determine the absorbance of the samples.

Western blotting. Protein samples were collected in RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS) containing 1x Protease Inhibitor Cocktail (Sigma-Aldrich) and protein content was quantified using a commercially available protein assay (BCA Protein Assay Kit; Pierce, Rockford, IL, USA) and a Biomate3 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). Samples were separated on 8-12% SDS polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Blocking was performed with 5% skim milk powder in Tris-buffered saline with 0.1% Tween-20 (TBS-T). For all subsequent immunoblotting, antibodies were diluted to the appropriate concentration in 5% skim milk powder in TBS-T. Blots were incubated with the following primary antibodies for 1 h at room temperature or overnight at 4˚C: rabbit-anti ATF3 (1:1000, C-19; Santa Cruz, Santa Cruz, CA, USA), rabbit-anti poly(ADP-ribose) polymerase (PARP) (1:1000; Cell Signaling Technology, Lake Placid, NY, USA), mouse-anti cyclin D1 (1:1000, A-12; Santa Cruz), rabbit-anti C/EBP homologous protein (CHOP) [also known as growth arrest DNA damage inducible gene 153 (GADD153)] (1:1000, R-20; Santa Cruz), and mouse-anti actin (1:10000; Sigma-Aldrich). Following three washes in TBS-T, blots were incubated with the appropriate horseradish peroxidase (HP)-labelled secondary antibody (goat anti-rabbit-HP, goat anti-mouse-HP, 1:5000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature in 5% milk in TBS-T. The chemiluminescent substrate used was Supersignal West Pico (Pierce) and the visualization of the protein bands was performed using the GeneSnap image acquisition system (Syngene, Frederick, MD, USA).

Chromatin immunoprecipitation (ChIP) assay. Cells treated for 24 h in 10 cm dishes were fixed in 1% formaldehyde (BDH; VWR International, Mississauga, ON, Canada) for 20 min at room temperature in order to cross-link the DNA and the protein and were evaluated as previously described by our group (20). Briefly, fixation was stopped by quenching with 2.5 mM glycine solution to a final concentration of 200 mM. Lysates were sonicated on ice using a Sonicator 3000 (Misonix; Farmingdale, NY, USA) at power setting #1 for a total of 3 min (10 s on/off pulses) to shear DNA to an average size of 300 to 1000 base pairs. Positive sample cell lysates were immunoprecipitated by overnight rotation at 4˚C with rabbit anti-ATF3 (1:200; Santa Cruz) primary antibody. Negative controls were incubated overnight with rotation at 4˚C in the absence of primary antibody. Immune complexes were collected by 2 h rotation at 4˚C with the addition of 40 µl protein A agarose/salmon sperm DNA 50% slurry (Millipore) to both positive samples and negative controls. The agarose beads were removed from the samples by centrifugation for 1 min at 3,000 rpm. The DNA–protein cross-links were reversed by overnight incubation with 100 µg proteinase K (Roche Diagnostics; Laval, QC, Canada) at 65˚C. DNA was purified using a QiaQuick PCR Purification Kit (Qiagen; Toronto, ON, Canada) according to the manufacturer's instructions. Quantitative PCR was performed using a Roche LightCycler Version 3 (Roche Diagnostics) with the following amplification conditions: 95˚C for 1 min, 40 PCR cycles of 95˚C

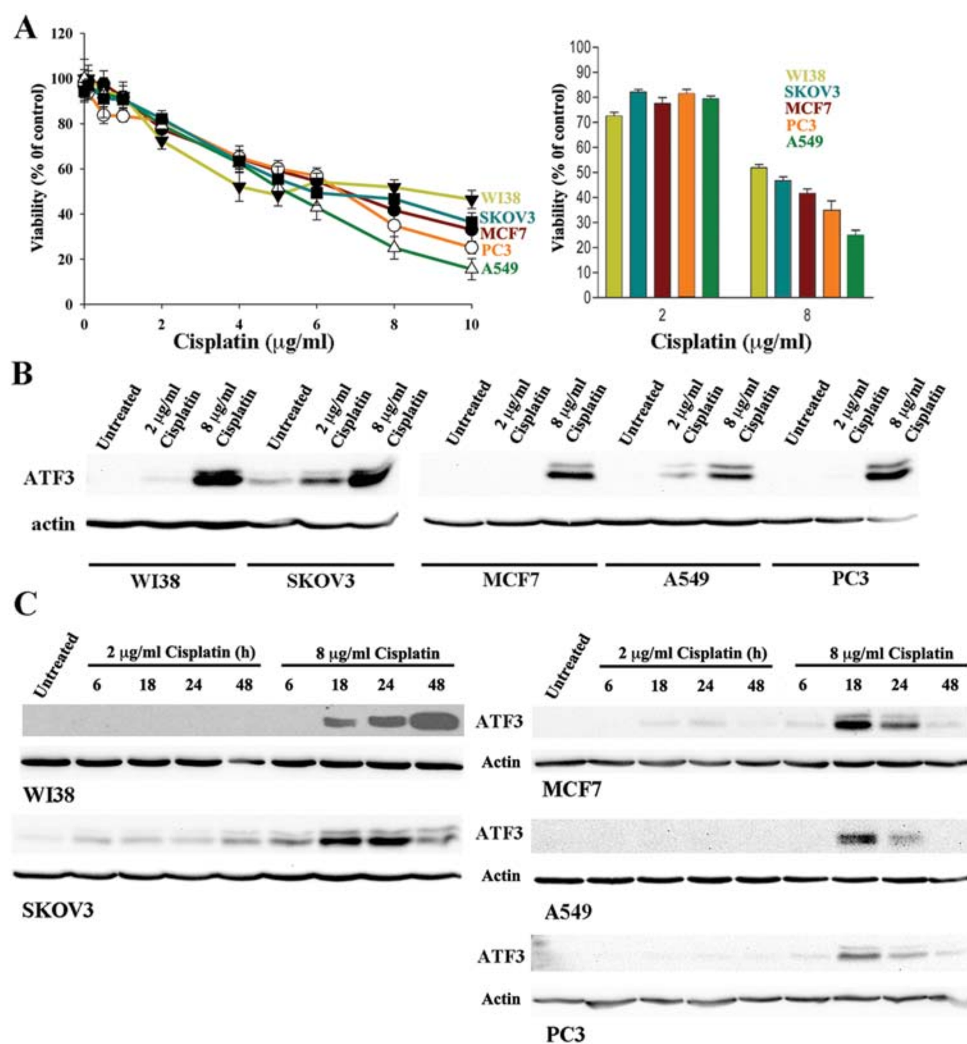

Figure 1. *ATF3 expression and cisplatin sensitivity in a panel of cell lines. A: MTT assay assessing the sensitivity of cell lines to increasing concentrations (0-10 μg/ml) of cisplatin over a 48 h period. Data are represented as the percentage of viability where untreated cells were taken to be 100%. Error bars are representative of six individual treated samples. B: Four cancer cell lines (SKOV3, MCF7, A549, PC3) and a normal lung fibroblast cell line (WI38) were treated with 2 μg/ml and 8 μg/ml cisplatin for 24 h. ATF3 protein was detected by western blot and actin was used as a loading control. C: Western blot analysis of ATF3 protein expression in WI38, SKOV3, MCF7, PC3 and A549 cells treated with cisplatin (2 and 8 μg/ml) at 6, 18, 24, 48 h time points. The cytotoxic dose (8 μg/ml) of cisplatin induces ATF3 protein expression in all five cell lines. Actin was used as a loading control.*

for 1 s, 58˚C for 10 s and 72˚C for 5 s. The binding of ATF3 to the *CHOP* gene promoter region was determined using the following primer pair: forward 5-GGCGGGACCCCAAAACCTACC-3' and reverse 5'-GCTCCTGAGTGGCGGATGCG-3'. PCR products were resolved on 1.6% agarose gels.

High-throughput chemical library screen. The PC3 and A549 cell lines were treated with a chemical library of 1200 FDA-approved compounds (Prestwick Chemical, Illkirch, France) (21). All compounds were supplied in a 10 mM stock diluted in DMSO and were used at a final concentration of 1 μM. The cell lines were exposed to the drug library alone for 72 h, or were pre-treated with the drug library for 24 h followed by cisplatin treatment (4 μg/ml) for 48 h. The MTT assay was used to determine cell viability as described above.

Evaluation of therapeutic interactions. The combination effect of disulfiram and cisplatin was evaluated by the Chou-Talalay method (22) using the CalcuSyn computer software (Biosoft, Cambridge, UK). The dose-effect curves of each drug alone, and in combination, were produced by the MTT assay. These data were entered into the CalcuSyn software, which derived combination index (CI) values that were graphed on fraction affected-CI (Fa-CI) plots. A CI<1 is a synergistic interaction, CI=1 is additive, and CI>1 is antagonistic.

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

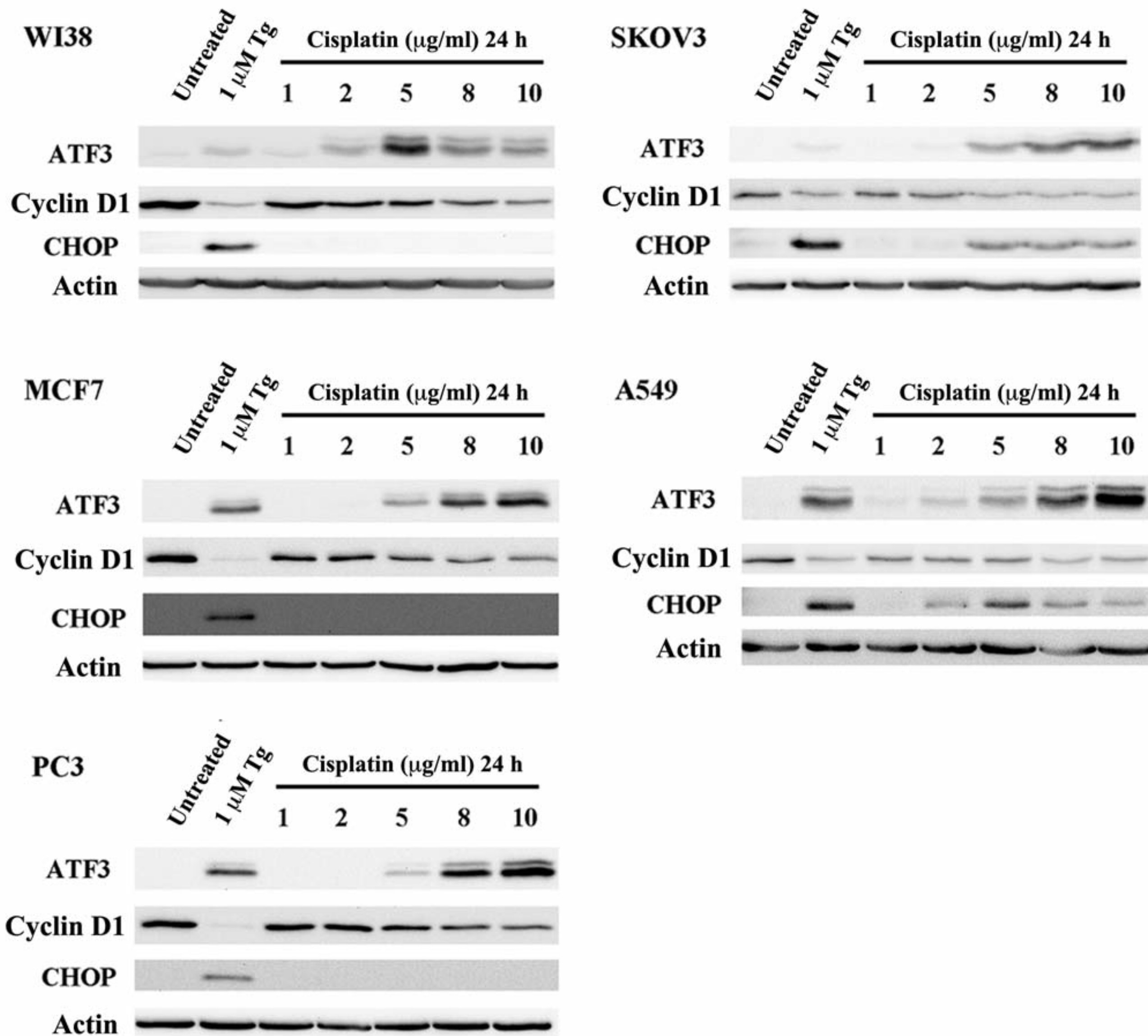

Figure 2. *Protein expression of potential ATF3 targets. Western blot analysis of ATF3, cyclin D1 and CHOP protein in a panel of cell lines following 24 h treatment with increasing concentrations (1-10 μg/ml) of cisplatin. Actin was used as a loading control and 1 μM thapsigargin, a known endoplasmic reticulum stressor, was used as a positive control. In the ovarian (SKOV3) and lung (A549) cell lines, cyclin D1 down-regulation correlated with ATF3 and CHOP up-regulation. In the breast (MCF7), prostate (PC3) and normal lung (WI38) cell lines cyclin D1 down-regulation is correlated with up-regulation of ATF3 but CHOP expression was not detected.*

Results

ATF3 protein expression induced by a cytotoxic cisplatin dose. In this study, we evaluated five established human cell lines that included WI38 (normal lung fibroblast), SKOV3 (ovarian adenocarcinoma), MCF7 (breast adenocarcinoma), PC3 (prostate adenocarcinoma), and A549 (NSCLC). The sensitivity of these cell lines to increasing concentrations of cisplatin (0-10 μg/ml) for 48 h was determined by the MTT assay (Figure 1A). Approximately 80% of cells survived a 2 μg/ml dose of cisplatin, whereas only 25-55% survival was observed with the 8 μg/ml dose of cisplatin. In all examined cell lines, basal ATF3 protein levels are either very low or non-detectable. Upon treatment with a cytotoxic dose (8 μg/ml) of cisplatin for 24 h, ATF3 protein expression was consistently up-regulated in all five cell lines examined (Figure 1B). In comparison, a sub-lethal dose (2 μg/ml) of cisplatin did not induce significant ATF3 expression.

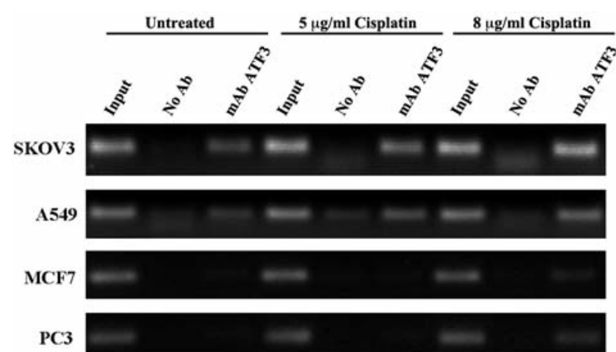

Figure 3. *ATF3 binds the CHOP gene promoter. ChIP assay of cancer cell lines treated with 5 μg/ml or 8 μg/ml cisplatin for 24 h. Formalin-fixed cell lysates were sonicated and incubated with an ATF3 antibody overnight. The antibody was pulled down using agarose beads, crosslinking was reversed and the DNA was purified. PCR amplification using primers specific to the CHOP gene promoter was performed and PCR products were run on 1.6% agarose gels.*

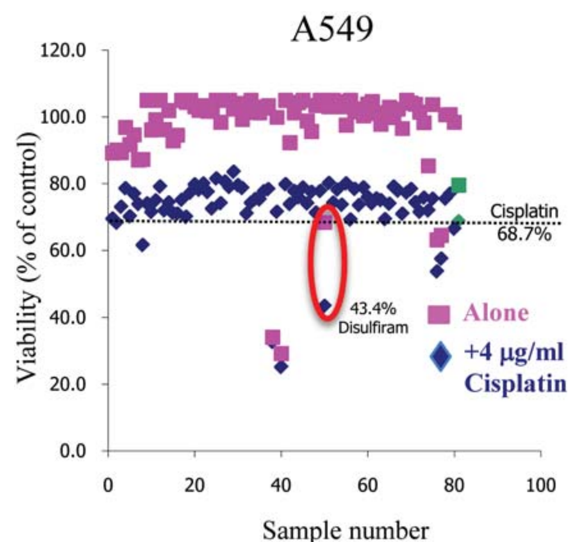

Figure 4. *Prestwick library screen for the identification of agents that enhance cisplatin cytotoxicity. PC3 and A549 cell lines were exposed to 1200 FDA approved compounds at 1 μM concentration for 72 h. At one compound per well, this screening amounted to 15×96-well plates. The cell line was pre-treated with the drug library for 24 h and then exposed to 4 μg/ml cisplatin for 48 h. Cell viability was assessed by the MTT assay where the untreated control cells were withheld as 100%. A selective hit is observed when the combination of a compound and cisplatin treatment kills more cells than cisplatin alone; an occurrence observed only a few times per plate (shown by red circle). This figure demonstrates a chemotherapeutic hit of disulfiram in A549 cells.*

A time-course experiment was conducted to gain insight into the kinetics of ATF3 protein expression upon cisplatin treatment (Figure 1C). ATF3 protein expression was evaluated by western blot at various time-points (6, 18, 24, 48 h) at both the non-cytotoxic (2 μg/ml) and the cytotoxic (8 μg/ml) doses of cisplatin. No significant ATF3 induction was detected with the non-cytotoxic dose of cisplatin in all cell lines except SKOV3, which has higher basal levels of ATF3. However, all five cell lines treated with the cytotoxic dose of cisplatin demonstrated readily detectable induction of ATF3. The normal fibroblast cell line WI38 showed its strongest induction at 48 h, whereas ATF3 expression in all of the cancer cell lines peaked at 18 or 24 h.

Downstream targets of ATF3 transcriptional regulation. As a transcription factor, ATF3 can modulate the expression of a variety of downstream targets that include the down-regulation of cell cycle regulators (23-26) and the up-regulation of apoptotic factors (27-29) that can play important roles in regulating the cytotoxic action of cisplatin in cancer cells. The cell cycle inhibitory and apoptotic effects of cisplatin may be mediated in part by ATF3’s binding and regulation of the *cyclin D1* and *CHOP* gene promoters, respectively. The expression of these ATF3 target genes in response to cisplatin treatment was determined by western blot analyses (Figure 2). Thapsigargin (1 μM, 24 h) was used as a control since it is known to activate both ATF3 and CHOP, and down-regulate cyclin D1 through the ISR pathway (27-30). ATF3 protein expression was increased in a dose-dependent manner upon exposure to cisplatin (1, 2, 5, 8, 10 μg/ml) for 24 h in all cell lines examined except WI38, which peaked at 5 μg/ml. Correspondingly, cyclin D1 protein expression was reduced at these same concentrations. Under similar experimental conditions, CHOP expression, a pro-apoptotic protein, increased only in the SKOV3 and A549 cell lines.

The lack of inducible *CHOP* expression may be the result of a relatively weak induction below the limit of resolution of these western blot analyses. To address this issue, we evaluated the potential of cisplatin-induced ATF3 to bind to the *CHOP* promoter in these cells. A ChIP assay was performed in these cell lines to determine if ATF3 specifically bound to the *CHOP* promoter following cisplatin treatments. In all cell lines examined, ATF3 was bound to the *CHOP* gene promoter upon the highest dose of cisplatin (8 μg/ml) (Figure 3). In both the cell lines that exhibited cisplatin-induced CHOP protein expression (SKOV3 and A549) and those that did not (MCF7 and PC3), *CHOP* promoter DNA was pulled down with the ATF3 antibody upon 8 μg/ml cisplatin exposure and to a lesser extent with 5 μg/ml cisplatin, and in the untreated control. Therefore, cisplatin-induced expression of ATF3 can inhibit cyclin D1 expression, and induce and bind to the promoter of *CHOP*.

Identification of disulfiram as an enhancer of cisplatin-induced cytotoxicity. We have recently demonstrated that cisplatin in combination with an ATF3-inducing HDAC

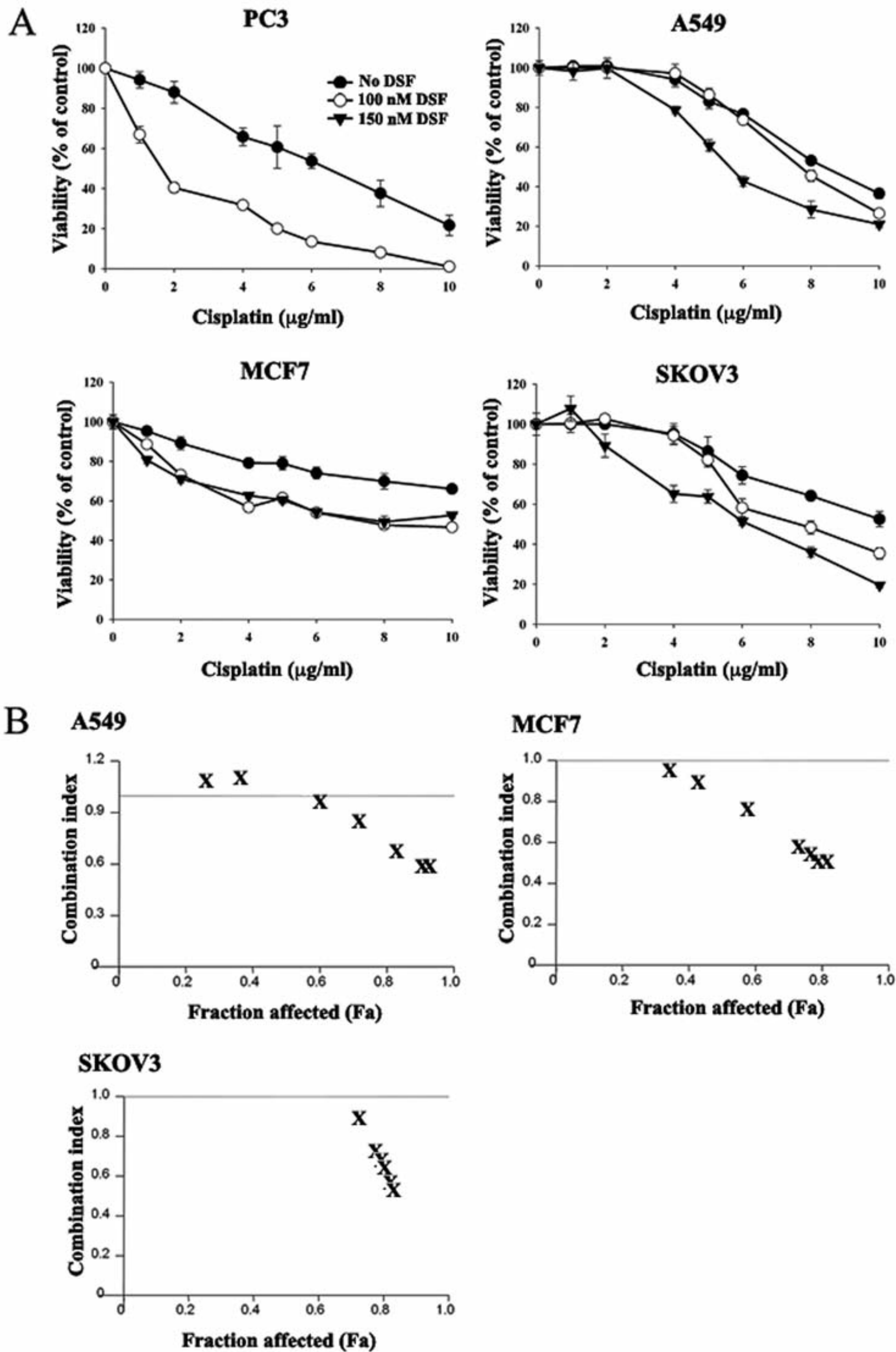

Figure 5. *Disulfiram enhances the cytotoxicity of cisplatin. A: MTT assays assessing cell line sensitivity to 24 h pre-treatment of 0 nM, 100 nM or 150 nM disulfiram followed by increasing concentrations (0-10 μg/ml) of cisplatin over a 48 h period. Differences in viability between cisplatin alone and 100 nM or 150 nM disulfiram combination treatment were statistically significant in all cell lines (one-way ANOVA, $p<0.001$). Data are represented as a percentage of viability where disulfiram alone (0, 100 and 150 nM) treated cells were taken to be 100% for ease of presentation. Error bars are representative of six individual treated samples. B: Combination effect of cisplatin and disulfiram was evaluated by the Chou–Talalay method using CalcuSyn software. Fa-CI plots represent the combination index values and the fraction affected at different concentrations of cisplatin (1, 2, 4, 5, 6, 8, 10 μg/ml) combined with 100 nM disulfiram. The additive effect of cisplatin and disulfiram is represented as CI=1; CI<1 indicates synergism; CI>1 indicates an antagonistic interaction. Cisplatin and disulfiram combination therapy results in synergy in all cell lines, over the higher doses of cisplatin evaluated.*

Table I. *Enhancers of cisplatin-induced cytotoxicity in A549 cells identified from a 1200 compound library screen (1 μM dose).*

Anti-metabolites	DNA intercalators	Topoisomerase I inhibitors	Microtubule polymerization	Other mechanisms
Methotrexate	Doxorubicin	Camptothecin	Docetaxel	Vorinostat (HDAC inhibitor)
Amethopterin	Daunorubicin	Topotecan		Disulfiram (alcoholism)
Floxuridine				
Gemcitabine				
Cladribine				

inhibitor M344 can augment the cytotoxic effects of cisplatin (20). Our goal is to identify more specific inducers of ATF3 expression that enhance the cytotoxicity of cisplatin tumour cell treatments. Thus, a high-throughput drug screen to identify potential enhancers of cisplatin cytotoxicity was performed. Two cancer cell lines, PC3 and A549, were treated with a chemical library of 1200 FDA-approved compounds, alone or in combination with cisplatin. Disulfiram, marketed as Antabuse and used for alcohol aversion therapy, increased the cytotoxic action of cisplatin in both cell lines examined (Figure 4, A549 results shown). As expected, MTT assay results showed increased cytotoxicity when cisplatin was combined with other chemotherapy agents such as topotecan, gemcitabine, daunorubicin and docetaxel (Table I), which are currently used in the treatment of ovarian cancer and NSCLC in combination with cisplatin. The HDAC inhibitor vorinostat also had a combined effect with cisplatin, validating our previous results in A549 cells (20).

This finding was validated in four cell lines by the MTT assay, using different concentrations of disulfiram (100 nM, 150 nM) and cisplatin (0-10 μg/ml) (Figure 5A). The combination of disulfiram and cisplatin greatly increased the cytotoxicity over that of cisplatin alone, and this was determined to be statistically significant (p>0.001) in all the tumour-derived cell lines examined. The graphs are normalized to disulfiram treatment alone for ease of presentation. To understand the nature of the combination effect, the Chou-Talalay method was used to distinguish between additive and synergistic interactions (22). Three out of the four cell lines were evaluated, since the PC3 cell line was too sensitive to the combination treatment to produce reliable results (Figure 5B). The potency and shape of the dose-effect curves of disulfiram and cisplatin alone, and in combination, were analyzed by the CalcuSyn software. Fa-CI plots were generated to show the combination effect. In all cell lines examined, the combination of 100 nM disulfiram and cisplatin was synergistic at high doses of cisplatin treatment.

Furthermore, we observed that disulfiram treatment up-regulated ATF3 protein expression (Figure 6A). In the PC3 cell line, ATF3 protein expression was enhanced with the disulfiram and cisplatin combination treatment. Disulfiram

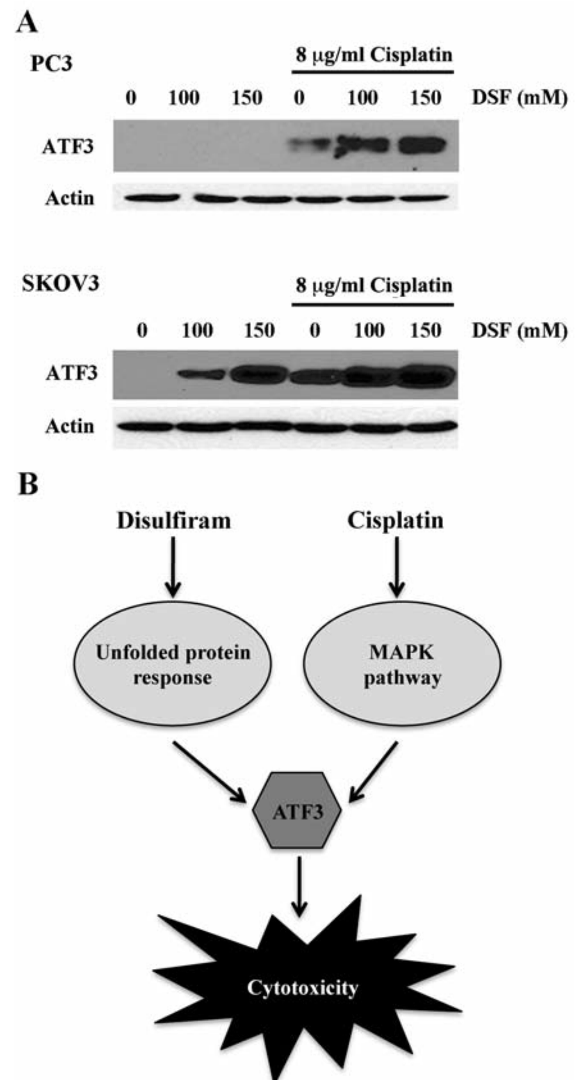

Figure 6. *Disulfiram induces expression of the ATF3 protein. A: Western blot analysis of the ATF3 protein upon treatment with 100 nM and 150 nM disulfiram (48 h), 8 μg/ml cisplatin (48 h), and pre-treatment with disulfiram (24 h) followed by concurrent disulfiram and cisplatin treatment (24 h). Actin was used as a loading control. B: Schematic model for enhancing cytotoxicity through ATF3. Disulfiram may up-regulate ATF3 though the unfolded protein response pathway and cisplatin up-regulates ATF3 though the MAPK pathway. Combinational treatment of disulfiram and cisplatin converge on ATF3, leading to enhanced cytotoxicity.*

alone up-regulated ATF3 protein expression in the SKOV3 cell line and ATF3 was also enhanced with the disulfiram and cisplatin combination treatment. Thus, disulfiram is a novel inducer of ATF3 expression and produced synergistic cytotoxicity with cisplatin in combination. This synergism is potentially due to the ability of these agents to enhance ATF3 expression through distinct mechanisms of action (Figure 6B).

Discussion

Cisplatin is a potent cytotoxic agent that induces cell death by directly damaging DNA. However, the mechanism of tumour cell toxicity is not fully understood. Various cellular pathways, such as the MAPK cascades, have been implicated in regulating tumour cell killing by cisplatin. With the goal of improving patient response to cisplatin and overcoming tumour resistance, the investigation of stress pathways may reveal novel therapeutic avenues. In a previous study, ATF3 up-regulation though the MAPK pathway was found to be an important regulator of cisplatin cytotoxicity (19). In our present study, ATF3 was shown to have a pro-apoptotic role in cisplatin response by directly binding to and activating the *CHOP* gene promoter, a key regulator of apoptosis. Cisplatin and disulfiram worked synergistically, in combination, to promote tumour cell death *in vitro*, and enhanced ATF3 protein expression was induced by this combination therapy. Overall, ATF3 plays an important role in the apoptotic response of these cytotoxic agents and it may serve as a potential indicator of treatment outcome in a clinical setting.

A cytotoxic dose of cisplatin induced ATF3 protein expression in a panel of cell lines. Previously, we demonstrated that ATF3 protein expression was localized in the nucleus of cisplatin-treated MCF7 and PC3 cells, consistent with its role as a transcription factor (19). In this study, ATF3 induction correlated with a reduction in cyclin D1 protein expression. This supports work by Lu *et al.*, which showed that ATF3 directly represses *cyclin D1*, leading to cell cycle arrest at the G_1–S checkpoint (23). ATF3 induction also correlated with an up-regulation of the pro-apoptotic protein CHOP in the SKOV3 and A549 cell lines and ATF3 was shown to directly bind the *CHOP* gene promoter in all four cell lines examined.

Our group screened a chemical library for compounds that synergized with cisplatin, and disulfiram emerged as a promising candidate. Disulfiram is a member of the dithiocarbamate class of chemicals, which forms complex with transition metals, used as vulcanizing and analytical agents in the field of chemistry (31). However, their use has expanded dramatically in the last few decades (31). Disulfiram was first used as a pesticide in the 1930s because it chelates copper, which is necessary for the respiratory chain of primitive animals (32). In the 1940s, it was marketed as Antabuse for the treatment of chronic alcoholism by preventing the complete metabolism of alcohol through the inhibition of human liver aldehyde dehydrogenase enzyme (31, 33). Disulfiram can also inhibit the metabolism of dopamine and is currently in development for the management of cocaine abuse (34). Long-term studies show that continued use of disulfiram is safe, with liver damage being the most common side-effect (Reviewed in 35).

Disulfiram was first shown to have an anticancer effect in the 1970s although its mechanism of action was unknown (36). Many cellular targets of disulfiram involved in multidrug resistance, angiogenesis and invasion have been described (37). However, its ability to inhibit the proteasome is of interest. In 2006, two studies demonstrated that the proteasome inhibitory effects of disulfiram induced suppression of tumour growth in breast cancer and leukaemia models (38, 39). This effect has been expanded to include efficacy in a wide range of cancer cells and patient-derived tumour cells, with disulfiram being most active in haematological, ovarian, breast and prostate cancers and NSCLC (40-42).

Since proteasome inhibitors are known to induce the ISR pathway (44), an upstream pathway of ATF3 induction, the involvement of ATF3 in disulfiram-induced apoptosis was investigated. ATF3 protein expression was greatly up-regulated upon combination treatment compared to disulfiram and cisplatin treatments alone. This suggests that ATF3 is induced by two separate pathways. The concept of synergism through ATF3 has been previously shown. An HDAC inhibitor that up-regulates ATF3 through the ISR pathway potentiated cisplatin cytotoxicity in a lung cancer model (20). ATF3 was important for cisplatin-induced cell killing since knockdown of *ATF3* by shNA attenuated cisplatin's toxicity. Moreover, overexpression of ATF3 by a tetracycline-inducible system enhanced the growth suppression of cisplatin, infrared radiation and etoposide (45). In addition, disulfiram has been shown to synergize with gemcitabine, which is also an activator of ATF3 (46, 47).

Therefore, including this study, there are a number of independent studies to support a role for ATF3 in the synergism observed between disulfiram and cisplatin shown here. Importantly, disulfiram appears to be selective for cancer cells due to their higher intracellular copper concentrations (38). This selectivity was shown in prostate cancer *versus* normal prostate epithelial cells (42), melanoma *versus* melanocytes (43), leukemia *versus* peripheral blood mononuclear cells (39), and breast cancer *versus* normal breast cells (38). Thus, the ability of disulfiram to inhibit the proteasome along with its safety profile makes it a viable anticancer agent.

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