

Role of the transcription factor Ets-1 in cisplatin resistance

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Abstract

Cisplatin is a DNA damaging agent widely used as a chemotherapeutic agent. A major limitation of the use of this agent is the development of drug resistance within tumors. Several *in vitro* models exist which enable the investigation of resistance mechanisms, including 2008/C13* ovarian carcinoma cells. C13* cells are variants of 2008 cells, displaying cisplatin resistance following 13 consecutive cisplatin treatments. This model system has led to the identification of several mechanisms that play parts in the multifactorial nature of cisplatin resistance. In this study, we have examined the contribution of a transcription factor, Ets-1, to the cisplatin resistance of C13* cells. Ets-1 is up-regulated in C13* cells as compared with the cisplatin-sensitive 2008 cells and overexpression of this protein in 2008 cells led to a 7-fold increase in resistance. Further studies on a colorectal carcinoma cell line overexpressing Ets-1 indicated that this phenomenon is not cell specific—increased cisplatin resistance correlated to Ets-1 expression. The mechanism of cisplatin resistance elicited by Ets-1 is potentially via transcriptional activation of genes whose products have well-described functions in reducing cisplatin toxicity. Examples, identified via microarray analysis, include metallothioneins and DNA repair enzymes. This is the first report to our knowledge associating expression of Ets-1, a transcription factor whose expression often signals poor prognosis in various cancer types, to cisplatin resistance. [Mol Cancer Ther 2004; 3(7):823–32]

Introduction

Platinum compounds, such as *cis*-platinum(II)-diammine dichloride (cisplatin), are DNA damaging molecules

widely used as anticancer agents (1). They have proved to be effective in the treatment of testicular and ovarian cancer as well as in combination for various other carcinomas (2). Cisplatin acts as a DNA alkylator and cross-linker, forming cross-links between guanine bases. These cisplatin-DNA adducts are presumed to initiate apoptosis in treated cells (3). The major limitation of cisplatin therapy is the development of drug resistance within tumor cells (1). Cisplatin resistance is frequently attributed to reduced adduct formation, decreased accumulation or increased efflux of the drug as well as enhanced repair of DNA adducts (4, 5). A limited ability to form adducts with DNA can partly be attributed to detoxification by thiol-containing agents that are able to coordinate with the drug and form a less toxic conjugate. As a result, increased glutathione as well as elevated metallothionein levels have been associated with cell resistance to cisplatin (6, 7). The major repair mechanism linked to cisplatin resistance is nucleotide excision repair, accordingly elevated levels of the rate limiting enzyme in this pathway (excision repair cross-complementing 1, ERCC1) is associated with resistance (4). Other DNA repair enzymes, such as thymidylate synthetase, an enzyme involved in the *de novo* pyrimidine biosynthetic pathway and essential for DNA replication and repair, and BRCA1—involved in transcription coupled nucleotide excision repair, have also been associated with cisplatin resistance (8, 9).

Though the above-mentioned mechanisms are well described in many *in vitro* models of cisplatin resistance, seldom does one mechanism completely account for acquired resistance, indicating that resistance is a multifactorial phenomenon. This is exemplified by the 2008/C13* human ovarian carcinoma cell model. 2008 cells were established from a patient with adenocarcinoma of the ovary and C13* cell variants were derived following 13 successive cisplatin treatments. 2008 cells are sensitive to cisplatin treatment, whereas C13* are 8- to 15-fold more resistant (10). Researchers have identified several determinants of cisplatin resistance within this model, including the reduced accumulation of cisplatin within C13* cells and the increased expression of DNA polymerase β in C13* variants (11, 12). DNA polymerase β seems to enhance translesion synthesis of platinum lesions and, thus, increases C13* cell survival following cisplatin treatment (11). Although it was originally reported that defective DNA repair does not account for cisplatin sensitivity in the 2008 cell line, as DNA mismatch repair and nucleotide excision repair capabilities are unchanged in the resistant cells (13, 14), recent studies have shown that the Fanconi Anaemia complement protein FANCF is not expressed in 2008 cells due to promoter methylation. In C13* cells, on the other hand, the promoter is demethylated and the protein expressed. Restoration of the expression of this DNA damage inducible protein, which plays a role in DNA repair, partially induces cisplatin resistance in the 2008 cells

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but does not completely account for the resistance of C13* cells (15). Other genes that have been observed to be up-regulated in C13* variants and, thus, may play a role in mediating resistance include those coding for dihydrodiol dehydrogenase, tropomyosin isoforms, apolipoprotein, glucose-6-phosphate dehydrogenase, and heat shock proteins (16, 17). In some cases, dihydrodiol dehydrogenase for an example, the overexpression of these proteins in 2008 cells induces partial cisplatin resistance, the mechanisms of which are not fully described (16, 17). Activity of the mitogen-activated protein kinase (MAPK) signaling pathways in these cell variants has also been studied; sensitivity to cisplatin seems to require a sustained activation of the JNK and p38 kinase enzymes that then lead to an up-regulation of Fas ligand and eventual cell death by apoptosis. The C13* cells do not display this prolonged activation (18). A final observation of note about the 2008/C13* model involves the mitochondria of these cells. It was observed that the mitochondria of the resistant cells are morphologically distinct and display an elevated mitochondrial membrane potential (19). Revertant cells selected by low membrane potential showed increased sensitivity to the drug (20). Though there are several lines of research describing resistance mechanisms in C13* cells, clearly there is no single mechanism. This model continues to generate data about potential mediators of cisplatin resistance.

Our lab has a particular interest in the role of transcription factors in the induction of cisplatin resistance. Several lines of evidence have indicated that the transcription factor *c-Fos* is associated with cisplatin resistance and this was examined in the 2008/C13* model (21). Although expression levels of this protein did not seem to differ significantly between the cells in question, *c-fos* antisense treatment in C13* cells led to increased cisplatin sensitivity, indicating that this transcription factor does play a role in the 2008/C13* model (21). *c-Fos*, as a constituent of the transcription factor activator protein-1 (AP-1), is known to act synergistically with another transcription factor, *Ets-1*, when bound to the tissue inhibitor of metalloproteinases-1 (TIMP-1) promoter. Presumably the two may act in synergy on other target gene promoters (22). It is, thus, possible that the *Ets-1* protein is of importance in the cisplatin resistance 2008/C13* model. In the work described here, we have revealed that the transcription factor, *Ets-1*, is up-regulated in C13* variants. *Ets-1* itself has been shown to transcriptionally up-regulate the expression of several genes involved in extracellular matrix remodeling and angiogenesis (23, 24). Expression of *Ets-1* in tumor and stromal cells has been correlated with poor prognosis in breast and ovarian cancer and is a predictor of tumor progression in certain cell models (25-28). We have investigated whether this transcription factor is also involved in cisplatin resistance by stably transfecting the sensitive, 2008 cells, with an *ets-1* plasmid, creating *Ets-1* overexpressing clones and subsequently determined cisplatin IC₅₀ values. Microarray analysis was conducted comparing gene expression in the parental 2008 and *Ets-1* overexpressing clones to

attempt to identify *Ets-1* targets that may account for cisplatin resistance. Similar experiments undertaken in an unrelated cell line, HT-29 colorectal carcinoma cells, support conclusions about *Ets-1* expression and cisplatin resistance. This is the first report indicating that the increased expression of *Ets-1* in C13* ovarian carcinoma cells may partially account for cisplatin resistance via the transcriptional activation of genes whose products have roles in mediating cisplatin resistance. *Ets-1* may be a new putative cellular target for the prevention of cisplatin resistance.

Materials and Methods

Cell Culture and Treatments

Human ovarian carcinoma cells (2008 and C13*) were provided by Dr. Paul Andrews, Georgetown University, Rockville, MD (19). COS-1 transformed monkey kidney fibroblasts were from American Type Culture Collection, Manassas, VA (ATCC CRL-1650) as were HT-29 colorectal adenocarcinoma cells (ATCC HTB-38). 2008 and C13* cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin solution, COS-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution, and HT-29 cells were maintained in MEM similarly supplemented. All cells were kept at 37°C in a humidified atmosphere of 5% CO₂. Media and supplements were purchased from Invitrogen/Life Technologies (Burlington, Canada). Cells were treated with various agents, including cisplatin and tetracycline (Sigma Chemical Co., Oakville, Canada).

Western Blot Analysis

Whole cell lysates were prepared and 50 µg of protein (determined using the Bradford protein assay) were separated by electrophoresis in SDS-10% polyacrylamide gel. Once separated, protein was transferred onto a nitrocellulose membrane and blocked with 5% skim milk in TBST for 1 hour. Membranes were then incubated overnight with a monoclonal mouse anti-*Ets-1* antibody (BD Biosciences, Mississauga, Canada). Following washing and 1 hour incubation in horseradish peroxidase-linked anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), proteins were detected by chemiluminescence (ECL, Amersham Biosciences, Baie D'urfe, Canada).

Plasmids

Human *ets-1* cDNA was obtained by reverse transcription-PCR on RNA extracted from C13* cells using gene specific primers. *ets-1* specific primers used, introducing flanking *Hind*III sites, were: forward: 5'-CCCAAGCTTATGAAGGCGGCCGTCGATCTC-3', reverse: 5'-CCCAAGCTTTCACCTCGTCGGCATCTGGCTTTAC-3'. pcDNA3-*ets-1* was created by cloning full-length *Hind*III *ets-1* cDNA into the *Hind*III site of a pcDNA3 expression vector (Invitrogen/Life Technologies, Burlington, Ontario, Canada). Constructs were screened for proper orientation and correct sequences. pcDNA6/TR encoding the tetracycline repressor protein

was from Invitrogen and the inducible *ets-1* plasmid, pcDNA4/TO/*ets-1*, was kindly provided by Dr. Hiro Yamamoto (The Chicago Institute of Neurosurgery and Neuroresearch, Chicago, IL) and has been described elsewhere (29).

Stable Transfection of Inducible *ets-1* Gene into 2008 Cells and Constitutively Expressed *ets-1* into HT-29 Cells

2008 cells were plated in a six-well tissue culture plate at 250,000 cells per well and incubated for 24 hours. Cells were then transfected with 2.0 μ g pcDNA6/TR by liposome-mediated transfer using Lipofectamine (Invitrogen/Life Technologies). Following 3 weeks of selection in 10 μ g/mL Blasticidin (Invitrogen/Life Technologies), surviving clones were isolated. Tetracycline repressor protein expression was evaluated. The surviving clone with highest expression of tetracycline repressor was then similarly transfected with the pcDNA4/TO/*ets-1* vector. Following 3 weeks of selection in 0.1 mg/mL Zeocin (Invitrogen/Life Technologies), surviving clones were isolated and tested for inducible *ets-1* expression (Ets-1 levels were evaluated following 24 hours of treatment with 2 μ g/mL tetracycline). HT-29 cells were similarly transfected with pcDNA3-*ets-1* (2 μ g) and treated for 3 weeks with 1 mg/mL Geneticin (Invitrogen/Life Sciences) at which time surviving clones were isolated and expanded.

Oligonucleotide Array Analysis

Total RNA was purified using the RNeasy mini kit (Qiagen, Valencia, CA) from 2008 (tetracycline induced for 24 hours), 2008-*ets-1*-5B, and 2008-*ets-1*-5B (tetracycline induced for 24 hours) cells. The relative abundance of specific RNA species was evaluated with the Human Genome Focus array (Affymetrix, Santa Clara, CA) that contains probes for the detection of approximately 8500 human gene sequences. Details for the preparation of biotinylated cRNA (generated from the RNA samples) and its use in probing the array is available from the array manufacturer (Affymetrix). Briefly, double-stranded cDNA was prepared from sample mRNA and made into biotinylated cRNA. This cRNA was then fragmented to fragments of 50 to 100 nucleotides that were hybridized with appropriate controls to the Affymetrix human genome focus array.

Data Analysis Using Affymetrix Software

Output files were analyzed using the Affymetrix microarray suite 5.0 software. Fluorescence intensity measured for each sample's chip was normalized to the average fluorescence for the entire group of samples (values were scaled to 150 so that chips could be directly compared). To identify differentially expressed transcripts, 2008 with tetracycline treatment was considered the baseline sample and 2008-*ets-1*-5B and 2008-*ets-1*-5B + tetracycline were separately considered experimental samples. Only genes with a significant detection in all three samples (considered significant based on a *P* detection value of <0.05) were considered in the identification of differentially expressed transcripts. Out of these transcripts, pairwise comparison

analysis was carried out (comparing the difference in values of each probe pair in the baseline array to the matching probe pair on the experimental array). A *P* change value was generated and changes were denoted as increase or decrease in gene expression. The fold change in signal intensity of genes in which a change was noted was calculated and only those with a 2 or greater fold increase in the highest Ets-1 expressor (2008-*ets-1*-5B + tetracycline) over the baseline (2008 + tetracycline) were considered as examples of increased gene expression.

Cisplatin Cytotoxicity Assay

For cisplatin cytotoxicity assays, 200 μ L of cells were seeded at 10,000 cells/mL in 96-well tissue culture plates and cisplatin was applied 24 hours later. Cisplatin treatment consisted of a 1-hour pulse followed by 96 hours recovery in cisplatin-free media at 37°C and 5% CO₂. The DNA binding bisbenzimidazo Hoechst 33258 (H 33258; Calbiochem-Novabiochem, San Diego, CA) is a fluorochrome used for the quantitation of cell number. Following treatment, cells were washed and lysed in milli-Q water and 2 μ g/mL H 33258 stock diluted in TNE buffer (10 mmol/L Tris, 1 mmol/L EDTA, 2 mol/L NaCl, pH 7.4) were added. Fluorescence was then evaluated using a Cytofluor series 4000 multiwell plate reader (PerSeptive Biosystems, Framingham, MA). Cell number was standardized to fluorescence for each cell type by comparison with a standard curve generated from seeding known cell numbers.

Statistical Analysis

Where appropriate, data were expressed as mean \pm SD based on at least three separate experiments. Student's *t* test was used for directly comparing data, with a *P* value of <0.05 considered significant.

Results

ets-1 Is a Cisplatin Responsive Gene

Having previously observed that *ets-1* mRNA levels are elevated in the C13* cells relative to 2008 cells (data not shown), Ets-1 protein expression was examined in the cells in question by Western blot analysis. It was confirmed that Ets-1 protein is expressed at a higher level in the C13* versus 2008 cells (Fig. 1). To probe the role of Ets-1 in the cellular response to cisplatin, a time course experiment was conducted to evaluate changes in Ets-1 expression

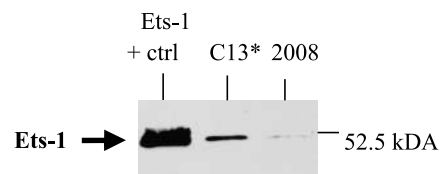


Figure 1. Level of Ets-1 in 2008 and C13* cells. Western blot analysis for the detection of Ets-1 protein levels in cell lines indicated. Fifty micrograms of whole cell lysates from each cell line were analyzed using a monoclonal antibody against Ets-1. Ets-1 is indicated at *M*, 51,000. Positive control (+ *ctrl*) used was 5 μ g of protein lysate from COS-1 cells transiently transfected with pcDNA3-*ets-1*.

following cisplatin treatment. 2008 cells—expressing a low basal level of Ets-1—were treated with various concentrations of cisplatin for 1 hour at which time medium was changed and cells were allowed to recover for 4 to 24 hours. An increase in protein expression was evident at 4 hours and dropped by 24 hours (data not shown). The Western blot shown shows the increase in Ets-1 protein levels in 2008 cells following cisplatin treatment with a range of concentrations at 4 and 6 hours recovery time (Fig. 2).

Overexpression of Ets-1 in 2008 Cells Increases Cisplatin Resistance

To investigate whether the up-regulation of Ets-1 in C13* cells is associated with cisplatin resistance, the cisplatin-sensitive 2008 cells were stably transfected with an inducible *ets-1* vector. 2008 cells were first transfected with a tetracycline repressor vector, pcDNA6/TR, and subsequently with the tetracycline inducible *ets-1* vector (pcDNA4/TO/*ets-1*). Following selection, the clones expressing the highest level of inducible Ets-1 protein were isolated for further analysis (2008-*ets-1-4* and 2008-*ets-1-5B*). Western blot analysis revealed that without tetracycline treatment, the tetracycline repressor protein was not capable of completely inhibiting transcription of *ets-1* from the inducible gene; levels of Ets-1 protein are increased in the un-induced clones as compared with the parental 2008 cells. Tetracycline treatment of these cells further enhances expression of Ets-1, thus, a range of Ets-1 expressors is available for analysis (2008, 2008-*ets-1-4*, 2008-*ets-1-5B* un-induced and both clones induced; Fig. 3A).

Cisplatin cytotoxicity profiles of 2008, C13*, and Ets-1 overexpressing clones were determined using a DNA binding agent (Hoechst) to assess cell number. Cells were treated with a 1-hour pulse of various concentrations of cisplatin and cell number was determined 4 days later. The cytotoxic responses to cisplatin of all cells tested are shown in Fig. 3B and C. Resistance of C13* cells relative to 2008 cells was confirmed (Fig. 3B, *inset*). Resistance of 2008 cells stably overexpressing Ets-1 was found to be greater than or comparable to C13* levels (Fig. 3B and C). The cisplatin IC₅₀ values were calculated and are shown on Table 1. Calculations indicate that C13* as well as Ets-1 overexpressing clones display significantly higher cisplatin IC₅₀ values than that of 2008 cells. Tetracycline induction significantly enhanced the cisplatin resistance characteristics of the *ets-1* tetracycline-inducible clone, 2008-*ets-1-5B*.

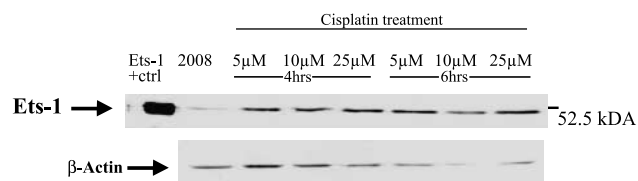


Figure 2. Up-regulation of Ets-1 by cisplatin treatment. 2008 cells were treated with 5 to 25 $\mu\text{mol/L}$ cisplatin for 1 hour, at which time medium was replaced and cells recovered for 4 to 6 hours. Lysates were made and 50 μg of whole cell lysates were analyzed by Western blot analysis using a monoclonal antibody against Ets-1. Protein levels were compared to that in untreated 2008 cells. Ets-1 is indicated at M_r 51,000.

However, cisplatin resistance of a second clone (2008-*ets-1-4*) was not significantly increased with further *ets-1* induction. Tetracycline treatment alone had no significant effect on the response of 2008 cells to cisplatin.

Overexpression of Ets-1 Induces Cisplatin Resistance in a Colorectal Carcinoma Cell Line

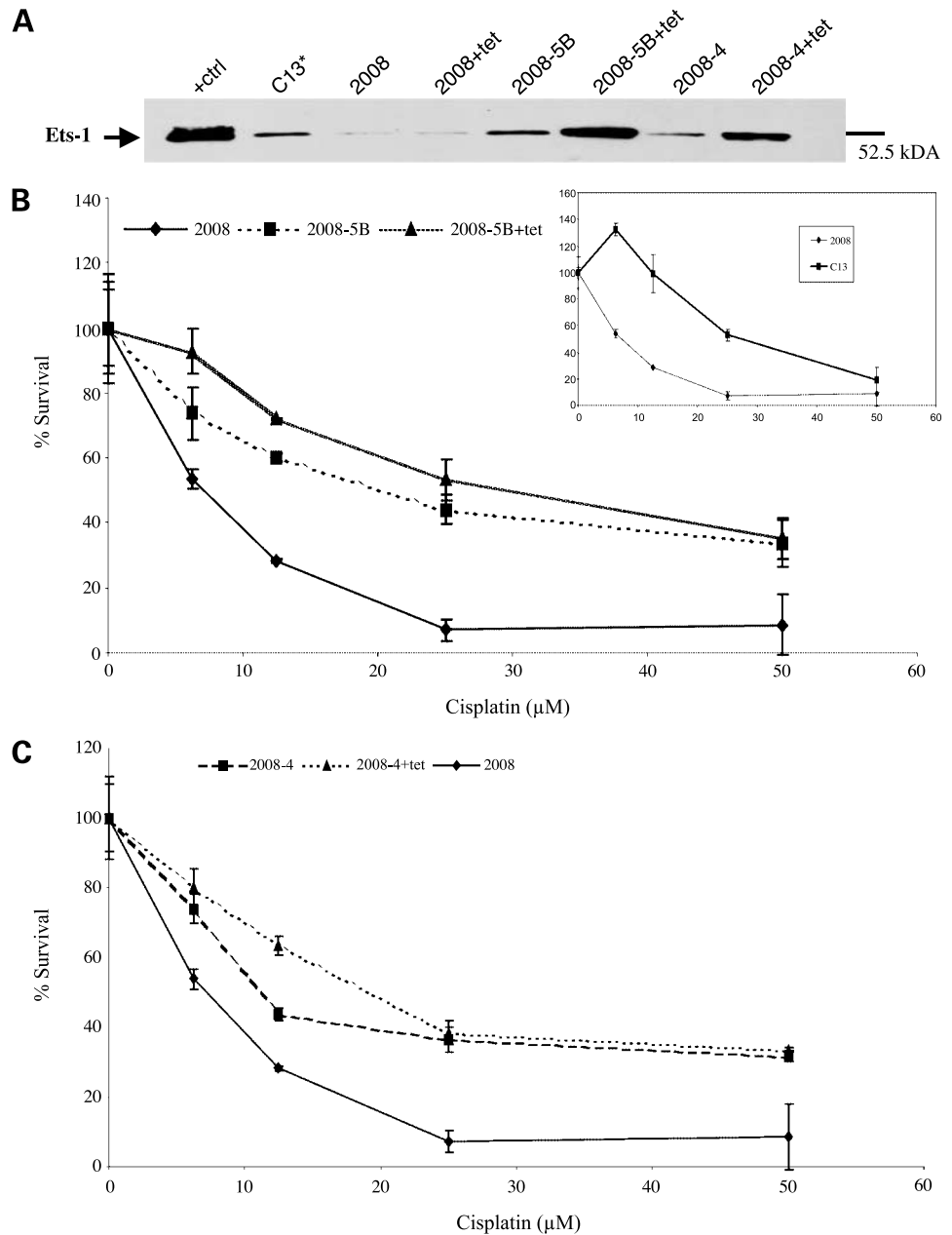
To verify whether the increased cisplatin resistance observed in 2008 cells following stable transfection with an *ets-1* vector is a cell-specific phenomenon, an unrelated, colon carcinoma cell line was similarly transfected with an *ets-1* vector. In this case, transfection with a constitutively expressed *ets-1* vector was done. Increased expression of Ets-1 protein was confirmed in clones 2 and 3 as compared with cells transfected with an empty vector (pcDNA3; Fig. 4A). Cisplatin cytotoxicity assays were done on HT-29 cells stably transfected with empty vector and *ets-1*. Survival curves indicate that HT-29-*ets-1-2* and HT-29-*ets-1-3* have greater survival rates at higher concentrations of cisplatin (Fig. 4B). IC₅₀ values confirm the statistically significant increase in the cisplatin resistance of Ets-1 overexpressing clones as indicated in Table 1.

Microarray Analysis Reveals Ets-1 Targets That May Account for Cisplatin Resistance

The ability of the transcription factor Ets-1 to induce cisplatin resistance in a sensitive cell line is likely via the transcriptional up-regulation of target genes involved in resistance mechanisms. To identify potential target genes, microarray analysis was done to compare gene expression in the parental 2008 cells and Ets-1 overexpressing clones. RNA was extracted from 2008-*ets-1-5B* as well as 2008 and 2008-*ets-1-5B* tetracycline induced for 24 hours. cDNA was synthesized from the RNA samples, made into biotinylated RNA and used to probe the Affymetrix Human Genome Focus array. Increasing levels of *ets-1* expression were verified in the microarray data—with a 3.6-fold increase in the 2008-*ets-1-5B* sample over basal (2008 + tetracycline) and a 27.3-fold increase in 2008-*ets-1-5B* with tetracycline treatment over basal. A total of 50 up-regulated genes was identified with at least a 2-fold increase in expression in the highest *ets-1* expressing cell line (2008-*ets-1-5B* + tetracycline) over the basal *ets-1* expression sample (2008 + tet). Among these are proteins involved in antioxidant defense, and, thus, potentially detoxification of cisplatin. Examples of such genes include metallothionein 2A and metallothionein 1X. Certain genes whose products are involved in DNA repair were also up-regulated, including the human homolog of mut Y and thymidylate synthetase. In each case, gene expression in all three samples tested correlated to the levels of Ets-1 within the cell lines (2008-*ets-1-5B* + tetracycline > 2008-*ets-1-5B* > 2008 + tetracycline; Table 2).

Array results detailing the expression of other genes whose products are potentially involved in cisplatin accumulation, repair of DNA adducts, and the induction of apoptosis were examined (Table 3). Although these targets were not picked up in the initial analysis, with the stringent criteria used to identify potential *ets-1* target

Figure 3. Effect of overexpression of Ets-1 on cisplatin resistance of 2008 cells. **A**, 2008 cells were stably transfected with pcDNA4/TR followed by pcDNA4/TO/*ets-1* to incorporate a tetracycline inducible *ets-1* gene. Western blot analysis for the detection of Ets-1 protein levels in lysates from samples is shown (+ *tet* indicates 24 hours tetracycline treatment with 2 $\mu\text{g}/\text{mL}$ tetracycline). **B**, representative cisplatin cytotoxicity curves for 2008 and C13* cells (*inset*) as well as 2008 Ets-1 overexpressing clones, 2008-*ets-1*-5B and 2008-*ets-1*-5B + tet (+ *tet* indicating cisplatin pulse was delivered following 24 hours of 2 $\mu\text{g}/\text{mL}$ tetracycline treatment). Cells were continuously treated with concentrations of cisplatin shown for 4 days at which time cell number was determined using the Hoechst assay to quantitate total DNA content. Cell number relative to cells grown in cisplatin-free media for 4 days was calculated at each concentration tested and percent survival is shown. *Points*, average of three samples in one representative experiment; *bars*, SD. Experiments were repeated three times yielding similar results. **C**, representative cisplatin cytotoxicity curves for 2008 cells overexpressing Ets-1 (2008-*ets-1*-4 and 2008-*ets-1*-4 + tet). Experiment was performed as described in **B**. Results were plotted along with those of 2008 cells for comparison purposes.



genes, this secondary analysis did indicate that DNA repair enzymes, such as BRCA2, BARD1 ERCC1, XPA as well as a gene previously linked to the cisplatin resistance of C13* cells (dihydrindiol dehydrogenase), tended to display higher hybridization signals in the *ets-1* overexpressing samples as compared with the 2008 sample.

Discussion

We have identified Ets-1 as a mediator of cisplatin resistance in the 2008/C13* cell model, extended this to a colorectal carcinoma cell line, and described possible

targets of this transcription factor that may account for resistance. Ets-1 has been investigated by many groups due to its ability to up-regulate genes whose products are involved in extracellular matrix remodeling or angiogenesis—the implication of these functions in tumor progression is of obvious interest (23, 24). This is the first report to our knowledge indicating that expression of Ets-1 in ovarian carcinoma may also play a part in tumor resistance to the chemotherapy agent cisplatin. Thus, the expression of Ets-1 in tumor cells may denote poor prognosis due to the high probability that the tumor has the capability to progress as previously suggested (27, 28) and also due

Table 1. Cisplatin IC₅₀ values

Cells	IC ₅₀ (μmol/L)
2008	4.87 ± 1.12
2008 + tet	7.79 ± 5.02
C13*	30.07 ± 8.43*
2008- <i>ets-1-5B</i>	19.23 ± 1.95*
2008- <i>ets-1-5B</i> + tet	37.84 ± 6.33* [†]
2008- <i>ets-1-4</i>	21.11 ± 7.30*
2008- <i>ets-1-4</i> + tet	21.70 ± 3.03*
HT29-pcDNA3	23.56 ± 3.29
HT29- <i>ets-1-2</i>	39.23 ± 6.92 [‡]
HT29- <i>ets-1-3</i>	37.41 ± 4.44 [‡]

NOTE: Cisplatin IC₅₀ values calculated for all cell lines tested are shown. Values represent the mean deviation from three separate experiments.

*Significantly greater than 2008 IC₅₀ value, $P < 0.05$.

[†]Significantly greater than 2008-*ets-1-5B* IC₅₀ value, $P < 0.05$.

[‡]Significantly greater than HT-29-pcDNA3 IC₅₀ value, $P < 0.05$.

to therapy resistance. Our data indicate that increased expression of Ets-1 in a cisplatin-sensitive ovarian carcinoma cell line induces up to 7-fold resistance. This surpasses the fold resistance of C13* cells we have noted in this study, but it should be pointed out that expression levels of Ets-1 in the most resistant clone (2008-*ets-1-5B* + tet) were higher than those of C13* cells. Furthermore, colon carcinoma cells stably transfected with an *ets-1* expression vector displayed altered cisplatin cytotoxicity profiles and increased IC₅₀ values. Though significant, this increase was not as

dramatic as observed in the 2008 cells, with a 1.7-fold increase in IC₅₀. This may be because expression levels of Ets-1 achieved in stable clones were not as high as those in 2008 transfectants and that the inherent cisplatin sensitivity of these cells is higher (increased expression of Ets-1 may only lead to increased resistance up to a certain threshold). This threshold effect may be due to autoinhibition by the transcription factor itself or perhaps by the transactivation of genes that counteract the effects of up-regulated gene products with roles in cisplatin resistance. This possibility may explain why in one 2008 *ets-1* overexpressing clone (2008-*ets-1-4*), enhanced expression of Ets-1 in the un-induced cells did increase cisplatin resistance by approximately 4-fold, whereas further increases in protein following tetracycline induction had no significant effect on resistance.

Several possible mechanisms of Ets-1 up-regulation in C13* cells exist. Ets-1 expression is up-regulated within stromal fibroblasts next to invasive tumors as well as within endothelial cells during tumor angiogenesis, indicating that a diffusible factor derived from tumor cells may be inducing Ets-1 expression. This is supported by studies showing growth factor mediated up-regulation of Ets-1 (30). Another plausible mechanism stems from observations that the *ets-1* gene is, in fact, a potential target for reactive oxygen species (ROS) and/or the hypoxia inducible factor 1α (HIF-1α) transcription factor (31, 32). In the ovarian carcinoma model described here, the mitochondria of the resistant cells are morphologically and functionally altered—Andrews and Albright (19) noted a higher

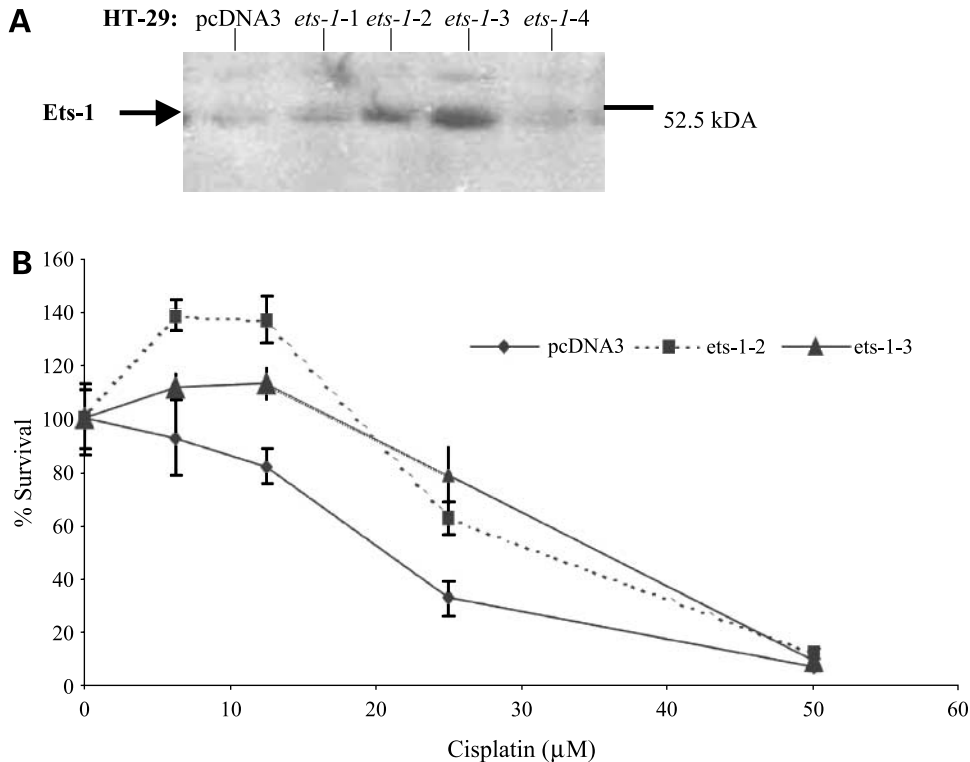


Figure 4. Effect of overexpression of Ets-1 on cisplatin resistance of HT-29 cells. **A**, HT-29 cells were stably transfected with pcDNA3-*ets-1*. Western blot analysis for the detection of Ets-1 protein levels in lysates from samples is shown. **B**, representative cisplatin cytotoxicity curves for HT-29 stable transfectants. Cells were continuously treated with concentrations of cisplatin shown for 4 days at which time cell number was determined using the Hoechst assay to quantitate total DNA content. Cell number relative to cells grown in cisplatin-free media for 4 days was calculated at each concentration tested and percent survival is shown. Points, average of three samples in one representative experiment; bars, SD. Experiments were repeated three times yielding similar results.

Table 2. Results of microarray analysis

Genes Up-Regulated in Cells Overexpressing Ets-1	Hybridization Signal			Expression Ratios	
	A: 2008	B: 2-5B	C: 2-5B + tet	B/A	C/A
FOS-like antigen-1	191.4	1,853.4	2,259.7	9.68	11.80
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2 (SERPINB2)	78.1	547.5	791.3	7.01	10.13
Diaphorase (NADHNADPH) (cytochrome <i>b</i> -5 reductase) (DIA4)	445.8	2,015.8	2,660.5	4.52	5.96
Tara mRNA	142.5	242.1	698.5	1.69	4.90
FXD domain-containing ion transport regulator 5 (FXD5)	208.1	830.4	1,014.4	3.99	4.87
UDP- <i>N</i> -acetylglucosamine-2-epimerase <i>N</i> -acetylmannosamine kinase (GNE)	37.6	116.6	152.5	3.10	4.05
Fatty acid desaturase 1	164	562.5	663.4	3.42	4.04
Metallothionein 2A (MT2A)	1,081.8	2,982.7	4,246.9	2.75	3.92
Retinal short-chain dehydrogenase reductase retSDR2	54.3	132.7	204.6	2.44	3.76
Macrophage myristoylated alanine-rich C kinase substrate (MACMARCKS)	112.5	322.1	396.1	2.86	3.52
Glutathione peroxidase 2 (gastrointestinal) (GPX2)	206.1	677	700.1	3.28	3.39
Similar to adaptor-related protein complex 1, σ 1 subunit	119.8	224.9	406.8	1.87	3.39
Thymidylate synthetase (TYMS)	1,952.8	5,507.6	5,900.8	2.82	3.02
Ribosomal protein L31	58.9	170.1	175	2.88	2.97
Craniofacial development protein 1 (CFDP1)	295.5	789.9	851.5	2.67	2.88
Cathepsin L (CTSL)	326.2	707.9	931.9	2.17	2.85
Metallothionein 1X (MT1X)	634.3	1,094.1	1,767	1.72	2.78
Katanin p80 (WD40-containing) subunit B 1 (KATNB1)	174	354	480.6	2.03	2.76
Glutamate-cysteine ligase, modifier subunit (GCLM)	352.6	960.9	968.1	2.72	2.74
PTPL1-associated RhoGAP 1 (PARG1)	267.7	680.7	715.6	2.54	2.67
1,2-cyclic-inositol-phosphate phosphodiesterase (ANX3)	184.2	471.1	490	2.55	2.66
ρ GDP dissociation inhibitor (GDI) β (ARHGDI β)	467.2	727.8	1,229.4	1.55	2.63
Similar to transforming growth factor β 1 induced transcript 1	87.9	215.5	229.1	2.45	2.60
Decay accelerating factor for complement (CD55, Cromer blood group system) (DAF)	64.8	115.7	168.5	1.78	2.60
Vaccinia related kinase 1 (VRK1)	335.6	779.2	863.5	2.32	2.57
Leman coiled-coil protein (LCCP)	178.7	444.8	458.5	2.48	2.56
Fasciculation and elongation protein ζ 2 (zygin II)	692.7	1,164.8	1,767.4	1.68	2.55
mutY (<i>E. coli</i>) homolog (MUTYH)	78.9	191.1	198.3	2.42	2.51
Fas (TNFRSF6)-associated via death domain (FADD)	109.4	189.5	273.6	1.73	2.50
Sperm autoantigenic protein 17 (SPA17)	106.4	205.1	262.2	1.92	2.46
hxCT mRNA for cystine glutamate exchanger	384	854.1	915.2	2.22	2.38
Sorting nexin 10 (SNX10)	109	240.1	256.2	2.20	2.35
SRY (sex determining region Y)-box 20 (SOX20)	477.4	923.9	1,118.1	1.93	2.34
<i>Homo sapiens</i> p35srj (MRG1)	57.2	88.7	133.9	1.55	2.34
Sm protein F (LSM6)	67.8	130.1	158.3	1.91	2.33
CCAAT enhancer binding protein (CEBP), δ (CEBPD)	113.5	216	260.7	1.90	2.29
Polycystic kidney disease 2 (autosomal dominant) (PKD2)	58	130	133.1	2.24	2.29
Protein phosphatase 1, regulatory subunit 10 (PPP1R10)	96.1	139.7	220.5	1.45	2.29
Lymphocyte adaptor protein (LNK)	49	100.5	109.5	2.05	2.23
Breast cancer anti-estrogen resistance 3 (BCAR3)	60.1	85.9	133.7	1.42	2.22
Target of myb1 (chicken) homolog-like 1 (TOM1L1)	246.2	507.1	542.2	2.05	2.20
Bifunctional ATP sulfurylase adenosine 5-phosphosulfate kinase	131.7	226.1	289.3	1.71	2.19
Crystallin, ζ (quinone reductase) (CRYZ)	278.3	580.8	604.2	2.08	2.17
Signal transducing adaptor molecule (SH3 domain and ITAM motif) 1 (STAM)	220.7	446.8	460	2.02	2.08
Photolyase	85.6	159.3	178.2	1.86	2.08
Thioredoxin	1,971.3	3,618.8	4,094.8	1.83	2.07
Serine threonine protein phosphatase catalytic subunit (LOC51723)	1,604.5	3,276	3,295.3	2.04	2.05
Ring finger protein 5	154	290.3	316	1.88	2.05
Non-ocogenic ρ GTPase-specific GTP exchange factor (proto-LBC)	177.7	256.9	363.4	1.44	2.04
Period (<i>Drosophila</i>) homolog 2 (PER2)	79.3	152.4	160.7	1.92	2.02

NOTE: Hybridization signals for genes that showed a significant detection in all three samples were considered in comparison analysis. Genes that showed a greater than 2-fold increase in signal in the highest Ets-1 expressor (2008-*ets*-1-5B + tet, 2-5B + tet in above table) over the baseline sample (2008 + tet) and were also observed to be increased in the 2008-*ets*-1-5B (2-5B) sample over the baseline are summarized. Hybridization signals in each sample as well as expression ratios (2008-*ets*-1-5B + tet/2008 + tet and 2008-*ets*-1-5B/2008 + tet) are shown. Bolded rows highlight genes with known roles in mediating cisplatin resistance.

Table 3. Further analysis of *ets-1* overexpression array

Mechanism of Increased Cisplatin Resistance	Gene	(A)	(B)	(C)	Expression Ratios	
		2008	2008- <i>ets-1</i> -5B	2008- <i>ets-1</i> -5B + tet	B/A	C/A
Reduced cisplatin accumulation	ATP7A	90.7	39	Absent	0.43	—
	ATP7B	91.4	82	77.8	0.90	0.85
	MDR1	Absent	Absent	Absent	—	—
DNA repair	Topoisomerase2	965	662.3	779.9	0.68	0.81
	BRCA I	299.7	413	469.7	1.38	1.57
	BRCA II	Absent	11.5	45.2	—	—
	BARD I	Absent	128.5	151.5	—	—
	ERCC I	157.5	286	206.3	1.82	1.31
	XPA	76.6	131	149	1.71	1.95
	hMSH2	167.3	193.2	140.7	1.15	0.84
	hMLHI	462.5	398.8	426	0.86	0.92
	DNA-Pol-β	356	233.7	Absent	0.66	—
Apoptosis	Fas	Absent	Absent	Absent	—	—
	Fas Ligand	Absent	Absent	Absent	—	—
	Caspase 3	107.8	119.4	88.4	1.11	0.82
	Caspase 8	101.3	135.3	195.8	1.33	1.93
	Caspase 9	88.2	116	96.9	1.31	1.10
	Bcl-2	Absent	Absent	Absent	—	—
Previously identified in 2008/C13* model	Bax	Absent	Absent	Absent	—	—
	FANCF	Absent	Absent	Absent	—	—
	Tropomyosin-(skeletal)	Absent	Absent	Absent	—	—
	Dihydrodioldehydrogenase	1,067.1	2,301.5	1,855	2.16	1.74

NOTE: Summary of array findings for genes with putative roles in mediating cisplatin resistance. Although none are considered significantly up- or down-regulated by analysis criteria, hybridization signals and expression ratios are shown. Absent indicates that hybridization signal was not detectable by this method.

mitochondrial membrane potential as well as less electron dense mitochondrial membranes and irregular, often absent cristae. We have confirmed these observations and also noted an elevated production of intracellular ROS in C13* cells (data not shown). The production of ROS is likely as a result of mitochondrial defects, resulting from a decreased ability to transfer electrons along the electron transport chain. Though this hypothesis requires further investigation, the up-regulation of *Ets-1* in C13* cells may be a downstream effect of alterations to the mitochondria that have already been implicated in cisplatin resistance.

A microarray was done on RNA extracted from 2008 cells and 2008 clones displaying increased expression of *Ets-1*—the purpose of this experiment was to identify putative targets of *Ets-1* that may account for cisplatin resistance. The array identified many transcriptional alterations, most with no obvious connection to cisplatin resistance; however, enzymes involved in antioxidant defense and DNA repair were recognized. Specifically, expression of two metallothionein genes correlated with *Ets-1* expression. Although there is no published evidence for the involvement of an *Ets* protein in the transcriptional control of metallothionein 2A, it has been observed that this gene is a target for *c-Fos* and, thus, may represent an

example of a promoter responsive to the synergistic activation by *c-Fos* and *Ets-1* (33). Thymidylate synthetase is of importance as the rate-limiting step in the de novo pyrimidine synthesis pathway. It has been reported that cisplatin inhibits the salvage pyrimidine biosynthesis pathway in lung cancer cells, thus, an up-regulation in this enzyme may compensate for such an effect (34). It is also of high interest that promoter studies on thymidylate synthetase regulatory elements have shown that a 20-nucleotide region containing an ETS binding element is sufficient and necessary for transcription and *Ets-1* is presumably involved in the regulation of this gene (35). There is also evidence for the involvement of *c-Fos* in regulating the thymidylate synthetase gene and, thus, this provides another possible target for the combined action of *c-Fos* and *Ets-1* (33). To further investigate other possible mechanisms of cisplatin resistance, such as decreased drug accumulation, enhanced nucleotide excision repair or base excision repair as well as decreased induction of apoptosis, the expression of genes whose products are implicated in these processes was examined on the array. Though these genes were not picked up in the initial analysis as being significantly up-regulated in the *ets-1* overexpressing clones, this second analysis did identify that there is a

trend for higher expression of other DNA repair enzymes in the *ets-1* overexpressing clones (such as BRCA1, BARD1, ERCC1, XPA). The expression of genes involved in cisplatin accumulation and apoptosis appeared to be unaltered. Expression of dihydrodiol dehydrogenase was also notably higher in the stable cell lines, a gene previously implicated in the C13* resistance model (16). The overall suggestion from array results is that there is an increase in thiol-containing molecules as well as up-regulated DNA repair enzymes in the *Ets-1* overexpressing cell lines that may mediate the cisplatin resistance of these cell lines.

It is apparent that the transcription factor *Ets-1* is a marker of poor prognosis in a range of tumor types (25, 26). This report reveals a possible mechanism that may underlie this observation. Importantly this finding may be of therapeutic significance as therapy aimed at inhibiting the actions of this transcription factor may be of use. This type of approach has been attempted with components of the activator protein-1 transcription factor as targets. *c-fos* antisense treatment increased cisplatin sensitivity in C13* cells and *c-jun* antisense was used in the A2780 human ovarian carcinoma cell line with derived cisplatin resistance (*c-jun* is a second component of the activator protein-1 complex; refs. 21, 36). In the A2780 model, investigators noted a 5-fold decrease in the cisplatin IC₅₀ of their resistant cells following antisense treatment (36). This has not, however, been attempted *in vivo* and in the case of *Ets-1*, there could be several limitations to this type of therapy. These include the likely inhibition of many other *Ets-1* target genes as well as potentially inhibiting the action of other *Ets* proteins that share sequence homology to *ets-1*. It may be a more reasonable approach to consider inhibiting the up-regulation of *Ets-1* by putative tumor-derived factors and, thus, studies of the regulation of *ets-1* are of obvious interest.

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