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Influence of the Proto-oncogene c-*fos* on Cisplatin Sensitivity

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ABSTRACT. Cisplatin resistance has been associated with overexpression of the c-*fos* gene in a human ovarian carcinoma cell line. To determine whether the correlation between c-*fos* overexpression and cisplatin resistance was limited to this cell line or was a more generalized phenomenon, we investigated cisplatin sensitivity in rat fibroblast cells that overexpressed the c-*fos* gene. The cisplatin IC_{50} values for two different c-*fos* transfectants, CMVc-*fos* and L1–3c-*fos*, were 7.6 ± 0.8 and 5.6 ± 1.0 μM, respectively, whereas the cisplatin IC_{50} value for the parental line, 208F, was 2.4 ± 0.1 μM. This represented a 3.2- and 2.3-fold resistance to cisplatin for CMVc-*fos* and L1–3c-*fos* cells, respectively. The correlation between c-*fos* expression and cisplatin resistance also was examined in a human ovarian carcinoma cell line, 2008, and its cisplatin-resistant variant, C13*. Expression of c-*fos* was elevated slightly at both the mRNA and protein levels in the C13* cells compared with 2008 cells, and c-Fos protein levels were induced in C13* cells following cisplatin treatment. In addition, it was observed that C13* cells were significantly more sensitive than 2008 cells to a c-*fos* antisense oligonucleotide. The IC_{50} values for the c-*fos* antisense oligonucleotide were 19.9 ± 5.0 pmol for C13* cells and 58.1 ± 6.0 pmol for 2008 cells ($P = 0.0012$). Furthermore, combinations of c-*fos* antisense and cisplatin reduced the amount of cisplatin required to kill 50% of the C13* cells, although the interaction was not synergistic. These results suggest that expression of the c-*fos* gene can influence cisplatin sensitivity, and that c-*fos* antisense oligonucleotide based therapy may be effective at killing parental and cisplatin-resistant ovarian carcinoma cells, either alone or in combination with cisplatin. BIOCHEM PHARMACOL **59**;4:337–345, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cisplatin; c-*fos*; resistance; antisense oligonucleotides; combination chemotherapy; ovarian carcinoma

Cisplatin is one of the most effective anticancer agents against a number of solid tumours, but it is limited by the frequent emergence of cisplatin-resistant cell populations [1, 2]. Resistance to cisplatin is particularly apparent in ovarian cancer, where the occurrence of cisplatin-resistant tumours approaches 60% [2]. Cisplatin resistance has been associated with a number of cellular changes, several of which have been investigated extensively, including: (i) enhanced scavenging of the drug through increased glutathione or metallothionein content, (ii) decreased accumulation and/or increased efflux of cisplatin, and (iii) increased repair of cisplatin–DNA lesions [3]. However, no single mechanism has been associated consistently with cisplatin resistance.

Several papers published by Scanlon and co-workers [4–6] indicate that cisplatin resistance is associated with overexpression of the proto-oncogene c-*fos*. The c-*fos* gene encodes a nuclear transcription factor, c-Fos, that induces transcription of a number of genes involved in the regulation of cell replication, cell cycle progression, and differentiation through its interactions with members of the c-Jun and ATF/CREB families [7–10]. Overexpression of the c-*fos* gene has been observed in tumour tissue from patients that have failed cisplatin-based chemotherapy and in human ovarian carcinoma cells selected for cisplatin resistance [2].

Several additional observations have strengthened the association between cisplatin resistance and overexpression of the c-*fos* gene. c-*fos* expression can be induced by physical stresses such as reactive oxygen species, heat shock, and several DNA-damaging agents, including cisplatin [11, 12]. Cells lacking the c-*fos* gene are hypersensitive to a number of DNA-damaging agents [13]. Transfection of a human ovarian carcinoma cell line with a c-*fos* expression vector induces cisplatin resistance [4]. Treatment of cisplatin-resistant human ovarian carcinoma cells with cisplatin induces the expression of the c-*fos* gene as well as a number of genes involved in DNA repair [5]. Reducing c-*fos* expression in these cisplatin-resistant human ovarian carcinoma cells through treatment with either cyclosporin A or a c-fos ribozyme results in decreased expression of the

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DNA repair genes dTMP synthase, topoisomerase I, and DNA polymerase β, and restores a large proportion of the cisplatin sensitivity [4–6].

In addition to restoring cisplatin sensitivity, it has been observed that reducing c-*fos* expression can, by itself, act as an effective strategy for treating tumours. Injection of MCF-7 cells containing a c-*fos* antisense retroviral vector into nude mice produced small, primarily encapsulated tumours that were not apparent until 10–12 weeks post-injection. In contrast, injection of MCF-7 cells containing a control vector into nude mice produced palpable tumours within 2 weeks, and these tumours invaded skeletal muscle and lymphatics [14].

To test the effectiveness of the c-*fos* antisense vector in inhibiting growth of established tumours, MCF-7 cells were injected i.p. and allowed to grow for 10 days. On day 10, either a control retroviral vector or the c-*fos* antisense retroviral vector was administered i.p. Administration of the c-*fos* antisense vector produced an 80% reduction in tumour mass compared with the control vector when examined 2 weeks after a single injection of the vector [14]. Furthermore, nine out of nine mice that received five daily doses of the c-*fos* antisense vector, 10 days after MCF-7 inoculation, survived for at least 55 days, whereas all nine mice receiving five daily doses of the control vector were dead by day 45. Based on these results, a clinical trial evaluating the effectiveness of c-*fos* antisense in metastatic breast cancer has been initiated [15].

Although exciting, the observed impact of c-*fos* on tumour growth and cisplatin resistance has been examined in only a limited number of systems, and thus it is difficult to determine whether the ability of c-*fos* to regulate tumour growth and/or cisplatin sensitivity is specific for these systems or whether the actions of c-*fos* extend to other systems. Therefore, we further investigated the role of c-*fos* in cisplatin resistance by examining the effect of c-*fos* overexpression on cisplatin sensitivity and by examining c-*fos* expression in a cisplatin-resistant variant. In addition, the potential of using c-*fos* antisense therapy in combination with cisplatin or as an antitumour agent on its own was examined in a human ovarian carcinoma cell line.

MATERIALS AND METHODS

Materials

cis-Diamminedichloroplatinum(II) (cisplatin), diethylpyrocarbonate, dithiothreitol, IPTG,* and phenylmethylsulfonyl fluoride were obtained from the Sigma Chemical Co. H33258 was obtained from Calbiochem. Rabbit polyclonal c-*fos* and cyclin D_1 primary antibodies were obtained from Santa Cruz Biotechnology. Goat anti-rabbit horseradish peroxidase conjugated secondary antibody was obtained from Bio-Rad Laboratories Ltd. RPMI medium, Dulbecco's modified Eagle's medium, lipofectin, and oligo$(dT)_{12–18}$ were obtained from Gibco BRL. GeneScreen Plus nylon filters were obtained from New England Nuclear Research Products. The human c-*fos* oligonucleotide probe was obtained from Oncogene Science, Inc. The phosphorothioate oligomer complementary to bases −6 to +14 of c-fos mRNA, FITC-labeled oligonucleotide, control phosphorothioate oligomer with no known complementary mRNA, and lipophilic cations were provided by Dr. B. Brown, Genta Inc.

Cell Lines and Culture Conditions

The rat fibroblast cell line 208F and its two c-*fos* transfectants, CMVc-*fos* and L1–3c-*fos*, were gifts from Dr. Tom Curran, St. Jude Children's Research Hospital. CMVc-*fos* cells contained a vector in which mouse c-*fos* gene expression was driven by the cytomegalovirus promoter; thus, they constitutively expressed high levels of c-Fos protein. L1–3c-*fos* cells contained a vector in which mouse c-*fos* gene expression could be repressed by the addition of IPTG [16]. CMVc-*fos*, L1–3c-*fos*, and 208F cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 mg/mL of amphotericin B; cells were maintained at 37° and 5% CO_2 in a humidified incubator.

The human ovarian carcinoma cell line 2008 and its cisplatin-resistant variant, C13*, were provided by Dr. Paul Andrews, Georgetown University. These cells were grown in RPMI medium supplemented with 5% fetal bovine serum, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 mg/mL of amphotericin B; they were maintained at 37° and 5% CO_2 in a humidified incubator.

Western Blot Analysis of c-Fos Protein

Cells in log-phase growth were seeded overnight in 20 × 100 mm culture dishes. After two washes with PBS, pH 7.4, cells were lysed with 500 μL of radioimmunoprecipitation assay lysis buffer (1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 1% phenylmethylsulfonyl fluoride, 3% aprotinin, and 1% sodium orthovanadate in PBS). Cells were scraped from culture dishes and transferred to microcentrifuge tubes. Samples were sheared with a 21-gauge needle, and phenylmethylsulfonyl fluoride (10 μL of a 10 mg/mL stock in isopropanol) was added to each tube. Following a 45-min incubation on ice, samples were centrifuged at 12,000 *g* for 20 min at 4°. Supernatants were transferred to fresh microcentrifuge tubes, and samples were stored at −80°. Protein concentrations were determined using the Pierce Micro BCA spectrophotometric protein assay [17].

Proteins (10–20 μg/lane) were separated by polyacrylamide gel electrophoresis: 3% stacking gel and 7.5% separating gel for c-Fos and c-Shc proteins, and 12% separating gel for cyclin D_1 protein. Proteins were stacked at 30 V for

* *Abbreviations*: CI, combination index; CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; IC_{50}, concentration of drug that inhibits growth in 50% of the cells; and IPTG, isopropyl β-D-thiogalactopyranoside.

30 min and separated at 100 V for 65 min. Proteins were transferred to nitrocellulose membranes using the Mini Trans-Blot Cell (Bio-Rad Laboratories Ltd.) at 100 V for 45 min. Membranes were blocked overnight in 1% skim milk and 1% BSA in TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20]. Western blot analysis was carried out using the appropriate rabbit polyclonal primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Protein levels were detected using enhanced chemiluminescence western blotting detection reagents (Amersham Canada Limited) and Kodak XAR 5 scientific imaging film (Eastman Kodak Co.)

c-fos *Antisense Treatment*

2008 and C13* cells were grown on coverslips placed at the bottom of the wells of a 6-well plate. To determine transfection efficiency, several lipophilic cations at different oligonucleotide:lipid ratios were tested. Cells were incubated for 4 hr in serum-free medium containing a FITC-labelled all-phosphorothioate oligomer (250 nM) in the presence and absence of different amounts of various lipophilic cations (lipofectin, lipofectamine, and two lipids generated by Genta Inc.). After the 4-hr incubation period, the oligomer-containing medium was removed, and cells were placed in supplemented medium for approximately 24 hr. Cells then were visualized under a fluorescent microscope. We observed that a 1:9 ratio of oligonucleotide: lipofectin produced positive staining in the nuclei of approximately 80% of both 2008 and C13* cells, whereas little or no nuclear staining was observed when cells were treated with the FITC-labelled oligonucleotide in the absence of lipophilic cation. In all subsequent experiments, cells were treated with the c-*fos* antisense oligonucleotide in a 1:9 ratio with lipofectin in serum-free medium for 4 hr.

Survival Assays

CMVc-*fos*, L1–3c-*fos*, or 208F cells in log-phase growth were seeded overnight in 24-well plates at a density of 2000 cells/well. Cells were exposed to cisplatin for 1 hr, after which drug-containing medium was removed, and cells were incubated in drug-free medium for 5 days at 37°. At this time the medium was removed, cells were washed once with PBS, and 300 μL of deionized water was added to each well of a 24-well plate. Then the DNA content of each well was determined as previously described [18, 19]. Cisplatin sensitivity also was assessed in 208F and CMVc-*fos* cells, using a colony-forming assay. In this assay, 208F and CMVc-*fos* cells in log phase growth were seeded in 6-well plates at a density of approximately 200 cells/well. Cells were treated with cisplatin for 1 hr and allowed to grow for 6 days in drug-free medium. Surviving colonies were stained with methylene blue, and colonies containing 20 or more cells were counted.

Logarithmically growing 2008 and C13* cells were seeded overnight in 24-well plates at a density of 5000 2008 cells/well and 2500 C13* cells/well. Cells were exposed to various concentrations of the c-*fos* antisense oligonucleotide for 4 hr, after which the antisense oligonucleotide was removed and the cells were allowed to grow for 5 days. Cell survival was assessed using a DNA fluorochrome assay as described above. To examine the interaction between cisplatin and c-*fos* antisense oligonucleotide, C13* cells were seeded overnight in 24-well plates at a density of 2500 C13* cells/well. Cells were treated with c-*fos* antisense (concentrations ranging from 9.75 to 312.5 pmol) in a 1:9 ratio with lipofectin in serum-free RPMI medium for 4 hr. Then cells were returned to fully supplemented RPMI medium, and various concentrations of cisplatin were added for 1 hr either immediately or 24 hr after c-*fos* antisense treatment. Cell survival was assessed 5 days after the initiation of c-*fos* antisense treatment, using a DNA fluorochrome assay as described above. The nature of the interaction between cisplatin and c-*fos* antisense was assessed using median effect analysis. CI values were calculated; a CI of 1 indicates that the two agents interact in an additive fashion, whereas CI values of <1 and >1 indicate that the two agents interact in a synergistic and antagonistic fashion, respectively [20].

RNA Extraction

Cells in log-phase growth were seeded overnight in 150 × 25 mm culture dishes at a density of 1×10^7 cells/dish. Then cells were exposed to an IC_{90} cisplatin concentration for 1 hr. RNA extraction using the method of Chomczynski and Sacchi [21] was initiated immediately, 1 hr, or 2 hr after the cisplatin treatment. RNA was stored in diethylpyrocarbonate-treated water at −20°.

Slot-Blot Analysis of RNA

RNA was denatured and heated for 15 min at 60°. Serial dilutions of RNA (maximum of 20 μg) in diethylpyrocarbonate-treated water were bound to GeneScreen Plus nylon filters using a slot-blot apparatus. Duplicate filters were generated; one was probed for c-fos mRNA, and the other was probed with an oligo$(dT)_{18}$ to determine the amount of mRNA blotted. The oligo$(dT)_{18}$ was labelled with [γ-^{32}P]ATP by the method of Maxam and Gilbert [22]; the human c-*fos* oligonucleotide was labelled with [γ-^{32}P]ATP according to the manufacturer's protocol. Slot blots probed with the oligo$(dT)_{18}$ were prehybridized in 15 mL of a solution containing 6x SSPE (20x SSPE contains 3 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA, pH 7.4), 5x Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), 1% sodium dodecyl sulphate, and 0.1% sheared salmon sperm DNA for 1 hr at 42°. Hybridization was carried out in 15 mL of a solution containing 5x SSPE, 5x Denhardt's solution, 0.005 M Na_2HPO_4, and 2 μg of ^{32}P-labelled oligo$(dT)_{12–18}$ for 1 hr at room temperature. Blots were washed four times for 5 min each at room temperature in 2x SSC (0.3 M sodium

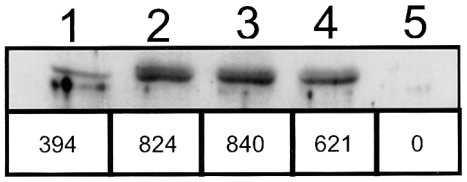

FIG. 1. c-Fos protein levels in 208F cells (lane 1), CMVc-*fos* cells (lane 2), CMVc-*fos* cells 24 hr after treatment with 2 mM IPTG (lane 3), L1–3c-*fos* cells (lane 4), and L1–3c-*fos* cells 24 hr after treatment with 2 mM IPTG (lane 5). Values below the western blot indicate the arbitrary densitometry values of the bands.

chloride, 0.3 M sodium citrate). Blots were placed in phosphoimaging cassettes, and the amount of radioactivity was determined on a phosphoimaging system (Molecular Dynamics).

Statistical Analyses

All values are means ± SEM unless otherwise indicated. Statistical significance was determined using an unpaired Student's *t*-test, and values of $P < 0.05$ were considered statistically significant.

RESULTS

c-fos Expression and Cisplatin Resistance in Rat Fibroblast Cells

Western analysis confirmed c-Fos protein levels in 208F, CMVc-*fos*, and L1–3c-*fos* cells. Basal c-Fos protein levels were examined in logarithmically growing cells and were found to be elevated in the CMVc-*fos* (lane 2) and L1–3c-*fos* (lane 4) cells compared with the parental 208F cells (lane 1, Fig. 1). Arbitrary densitometry readings below the western blot show that both CMVc-*fos* and L1–3c-*fos* cells had elevated c-Fos protein levels compared with the parental 208F cells. The c-*fos* expression vector in L1–3c-*fos* cells can be repressed by the addition of 2 mM IPTG [16]. Following IPTG addition to the L1–3c-*fos* cells, the level of c-Fos protein was reduced to undetectable levels (lane 5). As expected, IPTG had no effect on c-Fos protein levels in CMVc-*fos* cells, since the c-*fos* expression vector in these cells was unresponsive to IPTG (lane 3).

To evaluate the effect of c-*fos* expression on cisplatin sensitivity, these three cell types were treated with cisplatin (Fig. 2). The cisplatin IC_{50} for 208F cells was 2.4 ± 0.1 μM (N = 4); the IC_{50} values for CMVc-*fos* and L1–3c-*fos* cells were 7.6 ± 0.8 μM (N = 7) and 5.6 ± 1.0 μM (N = 6), respectively. Therefore, CMVc-*fos* cells were 3.2-fold resistant and L1–3c-*fos* cells were 2.3-fold resistant to cisplatin as compared with 208F cells.

To examine whether cisplatin sensitivity could be restored by the reduction of c-*fos* expression, L1–3c-*fos* cells were exposed to cisplatin in the presence and absence of 2 mM IPTG. As previously mentioned, IPTG was capable of repressing c-*fos* expression from the vector in the L1–3c-*fos* cells, and 2 mM IPTG was sufficient to reduce c-Fos protein levels (Fig. 1, lanes 4 and 5). The cisplatin IC_{50} for L1–3c-*fos* in the presence of IPTG was 7.2 ± 0.6 μM (N = 4) compared with 5.6 ± 1.0 μM in the absence of IPTG. However, a similar increase in the cisplatin IC_{50} from 7.6 ± 0.8 to 10.0 ± 1.0 μM was observed when CMVc-*fos* cells were incubated in the presence of IPTG. Since IPTG had no effect on c-Fos protein levels in CMVc-*fos* cells (Fig. 1, lanes 2 and 3) and in both instances the addition of IPTG increased the cisplatin IC_{50} about 1.3-fold, it appeared that IPTG somehow interfered with the cisplatin.

c-fos Expression in Human Ovarian Carcinoma Cells

In addition to showing that c-*fos* overexpression could lead to cisplatin resistance, we examined whether cells selected for resistance to cisplatin had elevated expression of the c-*fos* gene. For these experiments we used the human ovarian carcinoma cell line 2008 and its cisplatin-resistant variant, C13*. Although C13* cells have been reported to be as much as 12- to 13-fold resistant to cisplatin [23, 24], we consistently observed a 4- to 5-fold resistance to cisplatin (unpublished observations).

The amount of c-fos mRNA in 2008 and C13* cells was examined by slot-blot analysis using a human c-*fos* oligonucleotide labelled with [γ-^{32}P]ATP. c-fos mRNA levels were normalized for the amount of total mRNA loaded on the slot blot by probing a duplicate blot with a [γ-^{32}P]ATP-labelled oligo(dT)$_{12–18}$ probe. Although there was a small

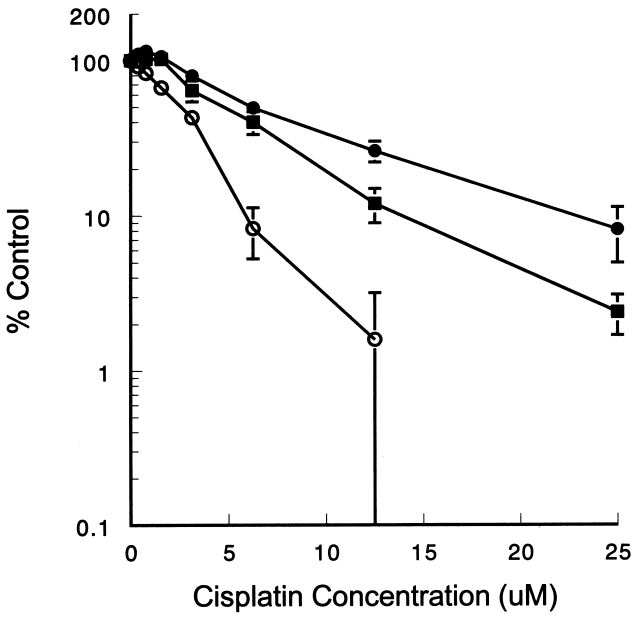

FIG. 2. Effect of cisplatin on (○) 208F, (●) CMVc-*fos*, and (■) L1–3c-*fos* cell survival. Cytotoxicity was assessed by a DNA fluorochrome assay 5 days after a 1-hr cisplatin exposure. Cells were seeded in 24-well plates at a density of 2000 cells/well, and untreated controls remained subconfluent 5 days later when cisplatin cytotoxicity was determined. Values are expressed as mean percent of control ± SEM, N = 4.

TABLE 1. c-fos mRNA levels following a 1-hr exposure to an IC_{90} cisplatin concentration

	c-fos mRNA levels	
	2008 Cells	C13* Cells
No cisplatin	0.55 ± 0.1	0.73 ± 0.1
Immediately after cisplatin treatment	0.65 ± 0.2	0.58 ± 0.2
1 hr after cisplatin treatment	0.46 ± 0.2	0.58 ± 0.3
2 hr after cisplatin treatment	0.50 ± 0.2	0.65 ± 0.3

Values (means ± SEM, N = 3) represent the amount of c-fos mRNA relative to the amount of total mRNA as detected by a ^{32}P-labelled oligo$(dT)_{18}$ probe (numbers are expressed as arbitrary units).

difference in the basal levels of c-fos mRNA between C13* and 2008 cells [c-*fos*:oligo(dT) ratio was 0.73 ± 0.1 (N = 3) for C13* cells and 0.55 ± 0.1 (N = 3) for 2008 cells], this difference was not significant ($P = 0.29$). The levels of c-fos mRNA also were examined following treatment of 2008 and C13* cells with their respective ic_{90} concentrations of cisplatin. Cisplatin treatment did not appear to alter the levels of c-fos mRNA noticeably in either cell line (Table 1).

At the protein level, C13* cells contained slightly more c-Fos than 2008 cells, a finding that was consistent with the mRNA levels (Fig. 3a). Treating 2008 cells for 1 hr with an IC_{90} concentration of cisplatin did not induce c-Fos protein levels at any of the time points measured (Fig. 3b). Treating C13* cells for 1 hr with an ic_{90} concentration of cisplatin induced a small increase in c-Fos protein levels at 2 and 4 hr after the cisplatin treatment, which then declined to below basal levels by 12 hr following the cisplatin treatment (Fig. 3c).

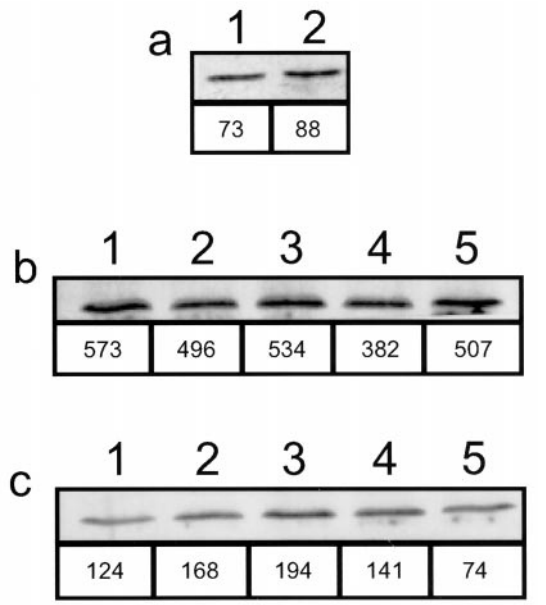

FIG. 3. Protein levels of c-Fos in (a) 2008 (lane 1) and C13* (lane 2) cells with no cisplatin pretreatment, and (b) 2008 or (c) C13* cells immediately (lane 1), 2 hr (lane 2), 4 hr (lane 3), 6 hr (lane 4), or 12 hr (lane 5) after a 1-hr incubation with an ic_{90} concentration of cisplatin. Values below the western blot indicate the arbitrary densitometry values of the bands.

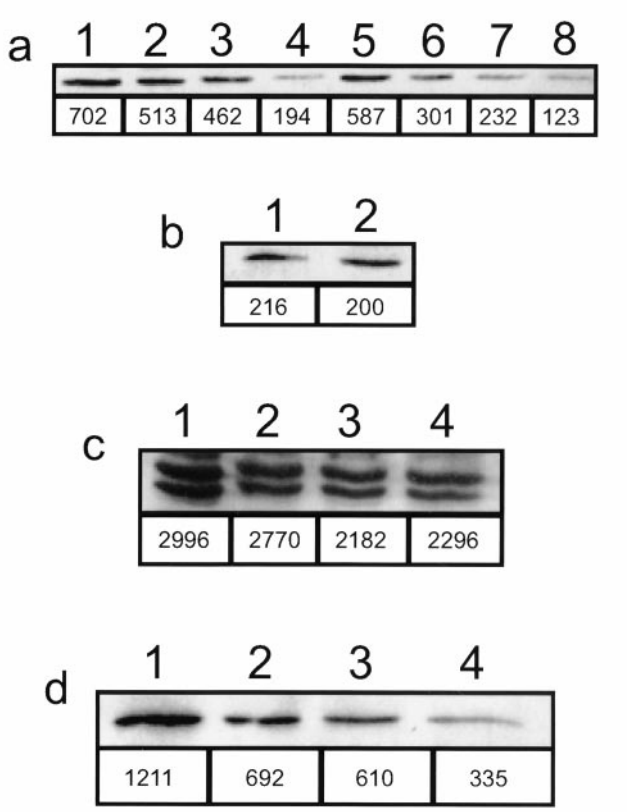

FIG. 4. Protein levels in C13* cells. (a) c-Fos protein levels 24 (lanes 1–4) or 48 (lanes 5–8) hr after c-*fos* antisense treatment: control (lanes 1 and 5), 156 pmol of c-*fos* antisense (lanes 2 and 6), 312.5 pmol of c-*fos* antisense (lanes 3 and 7), and 612.5 pmol of c-*fos* antisense (lanes 4 and 8). (b) c-Fos protein levels 48 hr after no treatment (lane 1) or treatment with a control oligonucleotide (lane 2). (c) c-Shc and (d) cyclin D_1 protein levels 48 hr after c-*fos* antisense treatment: control (lane 1), 156 pmol of c-*fos* antisense (lane 2), 312.5 pmol of c-*fos* antisense (lane 3), or 612.5 pmol of c-*fos* antisense (lane 4). Values below the western blot indicate the arbitrary densitometry values of the bands.

c-fos *Expression Following c-fos Antisense Treatment*

Although there were only slight differences in c-*fos* expression between cisplatin-sensitive and cisplatin-resistant cells, we still were interested in determining whether reduction of c-*fos* expression could be used to sensitize cells to cisplatin. To reduce c-*fos* expression, cells were treated with an antisense oligonucleotide directed against c-fos mRNA. Western analysis showed that c-Fos protein levels were reduced in a concentration-dependent manner that was apparent after 24 hr and remained low for at least 48 hr following a 4-hr c-*fos* antisense treatment (Fig. 4a). To investigate whether the addition of any oligonucleotide to C13* cells would affect c-Fos protein levels, C13* cells were incubated with a control oligonucleotide that has no known complementary RNA. At a concentration equiva-

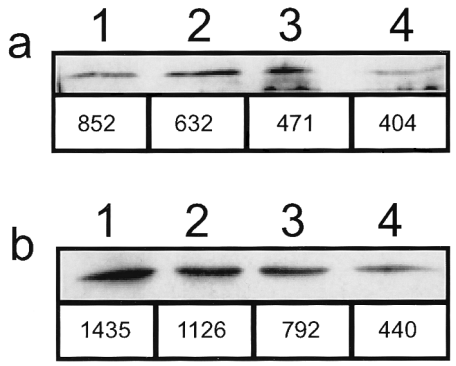

FIG. 5. Protein levels of (a) c-Fos or (b) cyclin D_1 in 2008 cells 48 hr after c-*fos* antisense treatment: control (lane 1), 156 pmol of c-*fos* antisense (lane 2), 312.5 pmol of c-*fos* antisense (lane 3), or 612.5 pmol of c-*fos* antisense (lane 4). Values below the western blot indicate the arbitrary densitometry values of the bands.

lent to the highest concentration of c-*fos* antisense used, 625 pmol, the control oligonucleotide did not appear to alter c-Fos protein levels (Fig. 4b). c-*fos* antisense also was shown to be specific for c-fos mRNA, since there was only a modest reduction in c-Shc protein levels following c-*fos* antisense treatment (Fig. 4c). However, the levels of cyclin D_1 protein, which has been reported to be regulated by c-*fos* expression [16], were decreased in C13* samples following c-*fos* antisense treatment (Fig. 4d). Similar results were observed in 2008 cells (Fig. 5a and b).

C13 Survival Following Combinations of c-fos Antisense and Cisplatin*

Combinations of cisplatin and the c-*fos* antisense oligonucleotide were performed to investigate whether combining c-*fos* antisense with cisplatin would restore cisplatin sensitivity in C13* cells. C13* cells were treated with the c-*fos* antisense for 4 hr and then treated with cisplatin for 1 hr either immediately after the antisense treatment or 20 hr after the end of the 4-hr antisense treatment. The interaction between cisplatin and c-*fos* antisense was determined by median effect analysis, and the graphs are presented in Fig. 6. In both cases the CI values were greater than or equal to 1, suggesting that cisplatin and the c-*fos* antisense interacted in an additive manner at best. However, combining the c-*fos* antisense with cisplatin reduced the amount of cisplatin required to kill the C13* cells almost to the level of the parental cells (Fig. 7). Using 19.5 pmol of c-*fos* antisense in C13* cells reduced the cisplatin IC_{50} from 14.3 to 5.5 μM, which was close to the 2008 cisplatin IC_{50} of 3.1 μM.

Interestingly, a cell survival assay indicated that treatment with the c-*fos* antisense inhibited cell survival in C13* cells to a greater extent than in 2008 cells (Fig. 8). The c-*fos* antisense IC_{50} was 19.9 ± 5.0 pmol in C13* cells and 58.1 ± 6.0 pmol in 2008 cells. Therefore, the cisplatin-resistant variant was significantly more sensitive to the c-*fos* antisense treatment than the parental line (2.9-fold, $P = 0.0012$).

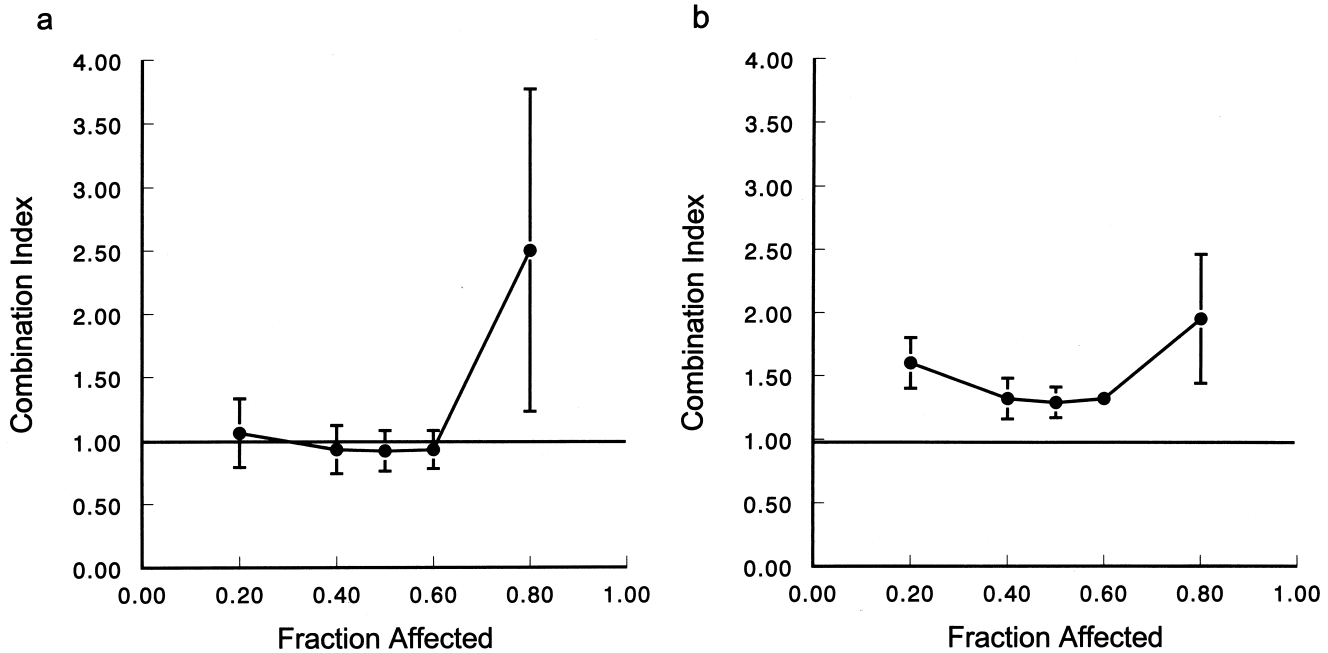

FIG. 6. Interaction of cisplatin and c-*fos* antisense in C13* cells. C13* cells, seeded in 24-well plates at a density of 2500 cells/well, were treated with various concentrations of cisplatin for 1 hr either (a) immediately after the c-*fos* antisense treatment or (b) 20 hr after the c-*fos* antisense treatment. Cell survival was determined 5 days later using a DNA fluorochrome assay. Untreated C13* cells remained subconfluent at the time when cytotoxicity was assessed. Values are expressed as means ± SEM, N = 3.

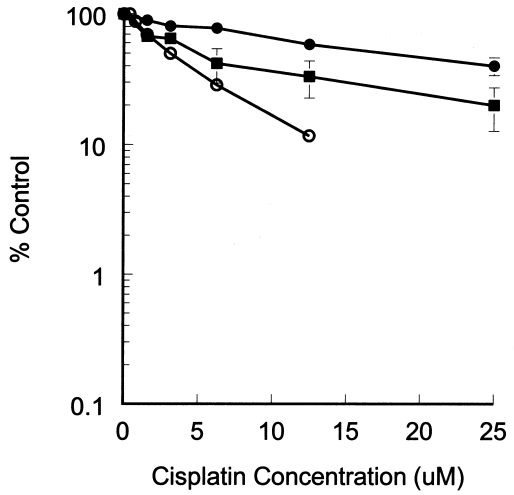

FIG. 7. Cisplatin survival curves following a 1-hr cisplatin treatment in (○) 2008 cells, (●) C13* cells, and (■) C13* cells pretreated for 4 hr with 19.5 pmol of the c-*fos* antisense oligonucleotide. Untreated controls for both cell lines were subconfluent when cytotoxicity, as assessed by a DNA fluorochrome assay, was determined 5 days later. Values are expressed as mean percent of control ± SEM, N = 3.

DISCUSSION

Several observations prompted the experiments described in this manuscript. First, elevated expression of the c-*fos* gene has been associated with cisplatin resistance in tumours from patients that did not respond to cisplatin-based chemotherapy and in established cisplatin-resistant cell lines [2, 25]. Second, reducing c-*fos* expression in cisplatin-resistant human ovarian carcinoma cells restores most of the cisplatin sensitivity [4–6]. However, these first two observations have been reported from only one group in one cell line and have not been confirmed or refuted. Finally, a retroviral vector that expresses c-*fos* antisense RNA is effective at reducing MCF-7 tumour size and incidence, and these findings have laid the foundation for a clinical trial investigating the effectiveness of this c-*fos* antisense retroviral vector against breast tumours [14, 15]. Therefore, the studies described herein examined whether c-*fos* overexpression was associated with cisplatin resistance in other cell lines and whether reducing c-*fos* expression was an effective method for increasing cisplatin sensitivity in cells resistant to cisplatin.

Overexpression of the c-*fos* gene using two different c-*fos* expression vectors induced 2- to 3-fold resistance to cisplatin compared with the parental rat fibroblast cell line. This observation is consistent with the finding that transfection of a human ovarian carcinoma cell line with the c-*fos* gene induces cisplatin resistance [5]. Since cisplatin sensitivity has been restored in a different cisplatin-resistant variant by reducing c-*fos* expression [4–6], several attempts were made to restore cisplatin sensitivity in the c-*fos* transfectants through reducing c-*fos* expression. Initially, the L1–3c-*fos* cells were treated with cisplatin in the presence and absence of 2 mM IPTG. Surprisingly, the IC_{50} for cisplatin was elevated in the presence of IPTG even though this concentration of IPTG was shown to reduce c-Fos protein levels. As a control, the CMVc-*fos* cells also were treated with cisplatin in the presence and absence of 2 mM IPTG. The expression vector in CMVc-*fos* cells did not respond to IPTG, as demonstrated in Fig. 1; however, the IC_{50} for cisplatin was elevated in CMVc-*fos* cells in the presence of IPTG as compared with CMVc-*fos* cells treated with cisplatin in the absence of IPTG. Thus, it appears that IPTG interfered with cisplatin-mediated cytotoxicity through a yet unidentified mechanism.

To overcome the potential inhibitory effects of IPTG on cisplatin, an attempt was made to reduce c-*fos* expression in CMVc-*fos* cells using an antisense oligonucleotide directed against c-fos mRNA. Oligonucleotide uptake is facilitated by the addition of cationic lipids such as lipofectin or lipofectamine. Efficiency of oligonucleotide uptake by cells was assessed using a FITC-labeled oligonucleotide and fluorescence microscopy. All three cationic lipids, lipofectin, lipofectamine, and a proprietary cationic lipid from Genta Inc., were ineffective at inducing oligonucleotide uptake by cells. Therefore, although we were able to demonstrate that c-*fos* overexpression could render cells resistant to cisplatin, we were unable to demonstrate that reducing c-*fos* expression could restore cisplatin sensitivity in the cisplatin-resistant c-*fos* transfectants.

In addition to investigating whether overexpression of c-*fos* was capable of modulating cisplatin sensitivity, cells selected for cisplatin resistance were examined for altered expression of c-*fos*. Cisplatin resistance has been associated with an elevation in c-*fos* expression in a different human ovarian carcinoma cell line, and it has been suggested that

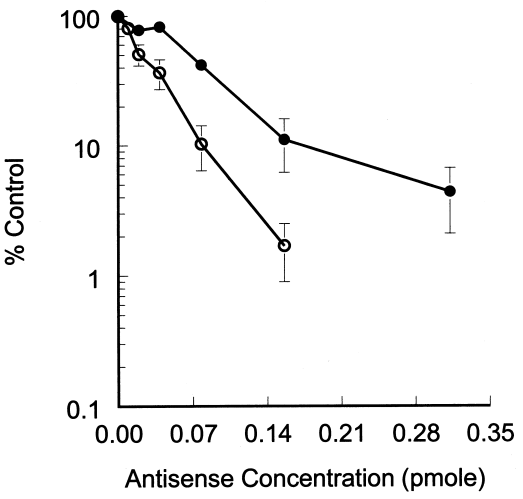

FIG. 8. Effect of c-*fos* antisense oligonucleotide on (○) 2008 and (●) C13* cell survival. Cells were seeded in 24-well plates at a density of 5000 cells/well for 2008 cells and 2500 cells/well for C13* cells. Cytotoxicity was assessed by a DNA fluorochrome assay 5 days after a 4-hr treatment with the c-*fos* antisense oligonucleotide. Untreated controls for both cell lines were subconfluent when cytotoxicity was assessed. Values are expressed as mean percent of control ± SEM, N = 3.

c-*fos* induces cisplatin resistance through enhancing the expression of several DNA repair enzymes including DNA polymerase β and topoisomerase I [4–6]. Moreover, C13* cells have been shown to repair cisplatin–DNA damage more efficiently than 2008 cells [26]. We observed a small increase in c-*fos* expression in C13* cells compared with 2008 cells at both the mRNA and protein levels. This increase in c-*fos* expression in the C13* cells relative to 2008 cells was considerably lower than the increase in c-*fos* expression previously reported in a different cisplatin-resistant human ovarian carcinoma cell line [4]. In addition, there was an increase in c-Fos protein levels in C13* cells but not in 2008 cells following cisplatin treatment that was not observed at the mRNA levels. This result is consistent with a previous report that cisplatin induces the expression of c-*fos* in cisplatin-resistant variants [4]. Therefore, there are modest differences between C13* and 2008 cells in both the basal levels of c-Fos protein and the ability of cisplatin to induce c-Fos protein.

To test whether reduction of c-*fos* expression in C13* cells could be used to restore cisplatin sensitivity, we used a c-*fos* antisense oligonucleotide to reduce c-*fos* expression in C13* cells. Protein levels of c-Fos could be reduced substantially by the addition of a c-*fos* antisense oligonucleotide as assessed by western analysis (Fig. 4a). Several experimental controls demonstrated that the c-*fos* antisense oligonucleotide was specific for c-fos mRNA: (i) addition of the c-*fos* antisense oligonucleotide decreased c-Fos protein levels in a concentration-dependent manner, (ii) a control oligonucleotide with no known complementary RNA sequence had no effect on c-Fos protein levels, (iii) the c-*fos* antisense oligonucleotide appeared to be specific for c-fos mRNA, as it did not affect c-Shc protein levels significantly, (iv) cyclin D_1, a protein reported to be regulated by c-*fos* expression [16], also was reduced following c-*fos* antisense treatment, (v) c-*fos* antisense treatment reduced cell proliferation in a concentration-dependent manner in both 2008 and C13* cells (data not shown), which is consistent with the ability of c-*fos* to regulate cell cycle progression [10, 27], and (vi) neither the c-*fos* antisense oligonucleotide alone nor the lipophilic cation alone was capable of decreasing cell proliferation (data not shown), suggesting that c-*fos* antisense uptake and localization in the nucleus were required for these effects.

The interaction between cisplatin and the c-*fos* antisense oligonucleotide was examined using median effect analysis. This analysis generates a graph in which the CI is plotted against the fraction of cells affected (Fa) by the treatment. CI values equal to 1 indicate that the two treatments interact in an additive fashion, whereas CI values greater than 1 and less than 1 indicate antagonistic and synergistic interactions, respectively. Combinations of cisplatin and the c-*fos* antisense produced CI values of 1 or greater in C13* cells, and thus the two agents did not induce synergistic cell kill. However, we were able to demonstrate that addition of the c-*fos* antisense to the cisplatin treatment in C13* cells reduced the amount of cisplatin required to kill 50% of the cells. Treating C13* cells with 19.5 pmol of the c-*fos* antisense reduced the cisplatin IC_{50} from 14.3 to 5.5 μM, which was close to the cisplatin IC_{50} for 2008 cells of 3.1 μM. Thus, although cisplatin and c-*fos* antisense oligonucleotides did not interact in a synergistic manner, the reduction in the cisplatin IC_{50} in cisplatin-resistant cells in the presence of the c-*fos* antisense still may be clinically relevant. The treatment of cisplatin-resistant tumours is limited by cisplatin-induced organ toxicity, and clinical resistance to cisplatin is generally in the range of 2- to 5-fold. Therefore, the addition of a c-*fos* antisense oligonucleotide to cisplatin-based therapy may benefit some patients.

It is difficult to compare the results obtained in this study with those demonstrating that cells transfected with a c-fos ribozyme completely restored cisplatin sensitivity in cisplatin-resistant human ovarian carcinoma cells [4]. In the studies by Scanlon *et al.* [4], it was shown that transfecting cisplatin-resistant cells with a c-fos ribozyme reduces the cisplatin IC_{50} to below that of the parental line. However, there has been no comment on the effect of the c-fos ribozyme on cell survival in the absence of cisplatin. Our results demonstrated that less cisplatin is required to kill cisplatin-resistant cells when combined with a c-*fos* reducing strategy, because inhibiting c-*fos* expression itself is lethal.

Probably the most surprising observation was that the cisplatin-resistant variant, C13*, was significantly more sensitive to the effects of the c-*fos* antisense alone than were the parental 2008 cells. The IC_{50} values for the c-*fos* antisense were 19.9 ± 5.0 and 58.1 ± 6.0 pmol for C13* and 2008 cells, respectively ($P = 0.001$). Enhanced sensitivity of C13* cells to a c-*fos* antisense oligonucleotide suggests that the modest increase in c-*fos* expression does not accurately reflect the importance of c-*fos* function in C13* cells.

The findings that the c-*fos* antisense oligonucleotide on its own is effective at killing both parental cells and cisplatin-resistant variants provide additional support for the clinical trial using a retroviral vector to express c-*fos* antisense in breast cancer patients [15]. In addition, results obtained in this study and others suggest that c-*fos* antisense therapy may be effective clinically against ovarian carcinoma. For instance, the retroviral c-*fos* antisense vector was effective at killing MCF-7 tumour cells grown i.p. [14]. Since ovarian tumours commonly grow confined to one area, and our study demonstrates that reducing c-*fos* expression kills both parental and cisplatin-resistant ovarian tumour cells, the use of the c-*fos* antisense retroviral vector may be an effective way to treat ovarian tumours, either alone or in combination with other agents.

In summary, overexpression of c-*fos* appears capable of rendering cells resistant to cisplatin. In addition, the cisplatin-resistant variant, C13*, had small increases in both c-fos mRNA and protein and was more sensitive to a reduction in c-*fos* gene expression than the parental line. Treatment of C13* cells with the c-*fos* antisense oligonucleotide reduced the amount of cisplatin required to kill

50% of the cells. These observations suggest that reducing c-*fos* expression represents an effective strategy for killing human ovarian carcinoma cells and/or cisplatin-resistant variants either alone or in combination with cisplatin.

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