Ribozyme-mediated reversal of the multidrug-resistant phenotype
(drug resistance/ovarian carcinoma)

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ABSTRACT This study examined the effects of suppressing c-fos oncogene expression on multidrug resistance (MDR). A2780S human ovarian carcinoma cells with resistance to actinomycin D were isolated and the resultant A2780AD cells exhibited the MDR phenotype. A hammerhead ribozyme designed to cleave for RNA cloned into the pMAMneo plasmid was transfected into A2780AD cells. Induction of the ribozyme resulted in decreased expression of c-fos, as well as that of the MDR gene (mdr-1), c-jun, and mutant p53. The transfectants displayed altered morphology and restored sensitivity to chemotherapeutic agents comprising the MDR phenotype. An anti-mdr ribozyme separately expressed in A2780AD cells efficiently degraded mdr-1 mRNA. However, reversal of the MDR phenotype by the anti-mdr ribozyme occurred one-fourth as rapidly as that induced by the anti-fos ribozyme. These results reinforce the central role played by c-fos in drug resistance through its participation in signal transduction pathways.

The development of drug resistance is one of the major obstacles in cancer chemotherapy (1). One of the most intensely scrutinized mechanisms is that of multidrug resistance (MDR), defined as resistance to a variety of different lipophilic compounds (2–4). Amplification of the multidrug-resistance gene (mdr-1) and resultant overexpression of the P-glycoprotein are thought to be chiefly responsible for development of MDR in many (5) but not all systems (6, 7). The P-glycoprotein is a 170-kDa transmembrane protein that functions as an energy-dependent efflux pump serving to decrease accumulation of structurally unrelated cytotoxic agents in the intracellular milieu (8).

The Fos oncoprotein has been implicated in a variety of cellular processes, including DNA synthesis, apoptosis, and drug resistance (9–11). Fos is thought to mediate its various effects through transcriptional activation after interaction with the Jun protein to form the AP-1 complex (12–14). Our previous studies have implicated c-fos overexpression in resistance to a number of chemotherapeutic agents, including cisplatin, 5-fluorouracil, and 3′-azido-3′-deoxythymidine (AZT), none of which is included in the MDR family (15, 16). We demonstrated that Fos may regulate DNA synthesis and repair pathways by upregulating transcription of a number of AP-1 responsive genes—namely, thymidylate synthase (17) and topoisomerase I (15). A ribozyme that cleaved c-fos mRNA was shown to reverse resistance to cisplatin, 5-fluorouracil, AZT, and camptothecin and to reduce expression of enzymes involved in synthesis of DNA and its precursors (15).

Interestingly, the upstream promoter of the mdr-1 gene contains an AP-1 binding site (18). In Chinese hamster ovary cells, this sequence is required for basal level transcription of the P-gp gene, which is homologous to the human mdr-1 gene. This suggests that the mdr-1 gene (and thus possibly the MDR phenotype) may be modulated by changes in c-fos expression. In this study, we examined the efficacy of ribozymes against c-fos and mdr-1 in reversing the MDR phenotype. Hammerhead ribozymes have demonstrated utility in suppression of gene expression in various model systems, including in vivo (15, 19–22). The anti-fos ribozyme was more efficacious than the anti-mdr-1 ribozyme in reversal of drug resistance. Moreover, evidence is presented suggesting that this reversal occurs through a transcriptional cascade brought about by repression of c-fos gene expression.

MATERIALS AND METHODS

Genes. cDNAs were graciously provided as follows: c-jun, R. Tjian (University of California, Berkeley); mutant p53 (p53-3c-1), M. Oren (Weizmann Institute, Rehovot, Israel); human topoisomerase I (clone D1), L. Liu (Johns Hopkins School of Medicine, Baltimore). Human c-fos (no. 41024) cDNA, human mdr-1 (no. 39839) cDNA, and the pMV M-Fos plasmid were obtained from American Type Culture Collection (Rockville, MD). cDNAs were isolated as described (15). [G-3H]Actinomycin D was obtained from Moravek Biochemicals (La Brea, CA).

A2780 Cells. The drug-sensitive human ovarian carcinoma cell line A2780S was obtained from R. Ozols (Fox Chase Cancer Center, Philadelphia), and the A2780AD-resistant cell line was isolated by weekly administrations of continuous exposure (for 72 hr) to actinomycin D for 9 months. In general, the cells were transferred to new RPMI 1640 medium on a weekly basis as described (15). The A2780AD cell line had a stable resistance to actinomycin D when grown in the absence of drug. For cytotoxicity determinations, 100 cells were inoculated in 60-mm tissue culture dishes. Twenty-four hours later, the cells were treated with cancer chemotherapeutic agents. The colonies were washed 6 days later, fixed in methanol stained with Giemsa dye, and assayed. A2780ADpfosRZ cells were pretreated with 0.5 μM dexamethasone for 24 hr before addition of the chemotherapeutic agents (15). The EC50 represents the drug concentration that inhibited 50% of the cell growth in the various cell lines.

Plasmid Construction. The anti-fos ribozyme was cloned into the plasmid pMAMneo (Clontech) as described (15). Primers for screening cell lines for the presence of pMAM-neofosRz plasmid were previously published (15).

The plasmid pHAAP-1 neo was obtained from L. Kedes (University of Southern California, Los Angeles). The anti-MDR ribozyme was prepared from two synthetic single-stranded oligodeoxynucleotides as described (19).

Transfection Studies. Subconflently growing cells were transfected by electroporation according to a protocol provided by IBI. Cells were propagated in medium containing geneticin (500 μg/ml) (G418 sulfat; Gibco) for 4 weeks. Individual G418-resistant colonies were picked, grown, and screened for expression of the anti-fos ribozyme. Reverse

Abbreviations: VP-16, etoposide; MDR, multidrug resistance; AZT, 3′-azido-3′-deoxythymidine; RT, reverse transcriptase.
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transcriptase (RT)-PCR, used to detect ribozyme expression, was performed with 100 ng of mRNA from various A2780 cell lines, primers for synthesis of the ribozyme construct, and a protocol provided by Perkin-Elmer/Cetus. The amplification, blotting, and hybridization procedures were performed as described (20). The sequences for primers and the probe used to detect anti-fos ribozyme expression were previously published (15). The quantification of the RT-PCR assay was performed by the concurrent use of known mRNA quantities in the amplification reaction.

**Northern Analysis.** RNA isolation, electrophoresis, hybridization, and densitometric analysis (AMBIS Systems) were performed as described (23).

**Transport Studies.** The uptake of radioactive [3H]actinomycin D (5 μM; 150 μCi/μmol; 1 Ci = 37 GBq; dissolved in RPMI 1640 medium without serum) into different A2780 cultured cell lines (60-mm-diameter Petri dishes) was measured as described (24). The uptake over a period of 120 min was quantified as follows: at each time point, cells were washed three times with ice-cold Dulbecco’s phosphate-buffered saline (PBS; without CaCl₂ and MgCl₂; Gibco). The radioactive material associated with these cells was solubilized by incubating the cells overnight in 1 M NaOH. Aliquots were saved for protein determination, and the remainder was neutralized with 1 M HCl. The radioactivity was determined by scintillation spectrometry as described (24).

**RESULTS**

Parental A2780S human ovarian carcinoma cells were grown in the presence of actinomycin D weekly for 9 months, and the resultant subline (denoted A2780AD) was shown to be 16.6-fold more resistant to actinomycin D, with an EC₉₀ of 10.0 nM, than were A2780S cells (EC₉₀, 0.6 nM; Table 1). A2780AD cells were demonstrated to exhibit the MDR phenotype, with cross-resistance to vincristine, doxorubicin, and etoposide (VP-16) (Table 2). Associated with this resistance spectrum was a morphological change to cuboidal cells when compared to the spindle-shaped drug-sensitive A2780S cells (Fig. 1). There was no increased resistance to methotrexate, a drug not in the MDR family.

Expression of the MDR phenotype was concomitantly associated with overexpression of the mdr-1 gene (Fig. 1), without the presence of mdr-1 gene amplification (data not shown). Moreover, [3H]actinomycin D uptake was shown to be significantly reduced in A2780AD cells (Fig. 2), corresponding to overexpression of mdr-1, which encodes the P-glycoprotein efflux pump. A2780AD cells were studied for expression of genes previously implicated in signal transduction and drug resistance pathways. Interestingly, A2780AD cells also overexpressed the protooncogenes c-fos and c-jun and, to a lesser degree, topoisomerase I and the mutant form of the tumor suppressor gene p53 (Fig. 1).

Elevated c-fos has been previously demonstrated in cisplatin-resistant cell lines (22), and expression of an anti-fos ribozyme reversed cisplatin resistance in A2780DDP cells (15). To investigate whether the anti-fos ribozyme could also modulate MDR, the dexamethasone-inducible plasmid pMAMneoRz (15) containing the ribozyme was electrooporated into A2780AD cells. Ten colonies were selected with resistance to G418, and five different clones were assayed and shown to express the anti-fos ribozyme and to have decreased c-fos mRNA (data not shown). Expression of the ribozyme in A2780ADpMAMRz (clone 2) cells was demonstrated.

### Table 1. Actinomycin D cytotoxicity in A2780 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EC₉₀, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780S</td>
<td>0.6</td>
</tr>
<tr>
<td>A2780AD</td>
<td>10.0</td>
</tr>
<tr>
<td>A2780AD (+ Dex)</td>
<td>8.5</td>
</tr>
<tr>
<td>A2780SpMMVfos</td>
<td>7.8</td>
</tr>
<tr>
<td>A2780AdpMAMneo (vector only)</td>
<td>9.0</td>
</tr>
<tr>
<td>A2780AdpMAMRz (no Dex)</td>
<td>0.8</td>
</tr>
<tr>
<td>A2780AdpMAMRz (+ Dex)</td>
<td>0.6</td>
</tr>
<tr>
<td>A2780AdpMAMRz (vector only)</td>
<td>9.7</td>
</tr>
<tr>
<td>A2780AdpMAMRz (vector only)</td>
<td>7.2</td>
</tr>
<tr>
<td>A2780AdpMAMRz (vector only)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

A2780 cells were plated and after 24 hr were treated with six different concentrations of actinomycin D by continuous exposure for 72 hr. Dexamethasone (0.5 μM) was administered as indicated (†Dex) to cells for 24 hr before drug treatment. A2780ADpMAMRz cells contained the pHJAp-1 neo plasmid without the ribozyme sequences. A2780AdpMAMRz cells transfected with a plasmid containing the anti-fos ribozyme in the reverse orientation. Results are means of triplicate sets of experiments.
strated by RT-PCR (Fig. 1). Basal level c-fos expression was reduced to \( \approx 15\% \) of control values in transformants (Fig. 1). After further induction of the ribozyme by dexamethasone administration in a time course assay, c-fos mRNA was maximally suppressed at 24 hr (Fig. 1; unpublished results). Fos protein expression was decreased in A2780ADpfosRz cells back to the sensitive level (data not shown). At the 24-hr time point, there was a concomitant decrease in expression of c-jun. mdr-1, topoisomerase II, and mutated p53 (Fig. 1). There was no significant change in expression of phosphoglycerate kinase in any of the cell lines or time points examined (Fig. 1; unpublished results).

Morphologically, A2780ADpfosRz cells resembled the A2780S cells in appearance with elongated, spindly cells (Fig. 1). Pharmacologically, sensitivity to actinomycin D was completely restored by ribozyme activation in A2780ADpfosRz cells, with an EC\(_{50}\) of 0.6 nM (Table 1). This was accompanied by reversal of resistance to other chemotherapeutic agents in the MDR phenotype, such as vincristine, doxorubicin, and VP-16 (Table 2). These results indicate that the anti-fos ribozyme reversed the MDR phenotype in A2780AD cells. Conversely, A2780S cells transfected with a vector containing the c-fos gene exhibited 13.0-fold greater resistance to actinomycin D (Table 1) and cross-resistance to agents in the MDR family (A2780SMMVfos cells; Table 2). As controls, A2780AD cells transfected with the pAMneo vector only and the anti-fos ribozyme in the reverse orientation (RfosRz) showed little change in resistance to actinomycin D (Table 1). Furthermore, 0.5 \( \mu \)M dexamethasone administered to A2780AD cells had no significant effects on actinomycin D cytotoxicity (Table 1).

Next, the effects of inhibiting mdr-1 gene expression on the MDR phenotype were investigated. To this end, the A2780AD cells were transfected with the pHdmrd1 plasmid containing the \( \beta \)-actin gene promoter and encoding a ribozyme previously designed and demonstrated to cleave the GUC sequence at codon 880 of exon 21 of mdr-1 mRNA (ref. 34; Fig. 3A). This target site resides between two ATP-binding sites, with possibly important implications for P-glycoprotein function (8). The resultant A2780ADpmdmrRz cells were shown to express the anti-mdr ribozyme by RT-PCR (Fig. 1). mdr-1 gene expression was reduced by >90% in A2780ADpmdmrRz cells when compared to A2780AD cells (Fig. 1). Demonstration of ribozyme cleavage was achieved by Northern analysis of mdr-1 polyadenylated mRNA. In Fig. 3B, using 2 \( \mu \)g of cellular mRNA and labeled mdr-1 cDNA, only the full-length mdr-1 transcript (4.3 kb) was detected in A2780AD cells (lane 2). In A2780ADpmdmrRz cells (lane 3), no mdr-1 transcript was detected in 2 \( \mu \)g of mRNA.

**DISCUSSION**

This manuscript has further examined the role of the c-fos gene in drug resistance by demonstrating that (i) cells expressing the MDR phenotype overexpress c-fos; (ii) cells...
transfected with and overexpressing c-fos exhibit MDR; and (iii) an anti-fos ribozyme reversed the MDR phenotype in A2780AD cells. Our data utilized morphological, pharmacological, and molecular analysis to better define this association. Fos has been previously shown to play a role in resistance to agents not within the MDR family, such as cisplatin, AZT, and 5-fluorouracil (15). Taken together, these studies indicate an expanded and more significant role for overexpression of c-fos in resistance to many of the different chemotherapeutic agents currently in use. Moreover, these results may have important implications for understanding the acquisition of drug resistance in oncogene-transformed cancer cells. Our results also suggest that Fos mediates some of these effects through transcriptional activation of AP-1 responsive genes, such as mdr-1, topoisomerase I, metallothionein IIA, and thymidylate synthase. The observation that c-jun expression is also decreased in A2780AD fosRz cells supports the hypothesis that the aforementioned effects may occur through Fos-Jun interaction. Our results indicate that activation of this transcriptional cascade is important in MDR because anti-fos ribozyme action diminished downstream gene expression at 24 hr, the time point at which EC50 values were measured.

Expression of mdr-1 has been previously demonstrated to be modulated by the Ha-ras and p53 genes in chloramphenicol acetyltransferase assays (25, 26). These studies used cotransfection assays with the downstream promoter cloned into the chloramphenicol acetyltransferase-containing vector in order to assess promoter responsiveness to these genes. We analyzed gene expression in a cellular environment and in a time-dependent fashion after ribozyme induction by dexamethasone. These results support the previous finding linking mutant p53 expression with that of mdr-1. They extend that concept by demonstrating that diminished p53 and mdr-1 mRNA after anti-fos ribozyme action may contribute to reversal of the MDR phenotype. These studies also suggest an association between expression of c-fos and p53. Moreover, a putative connection exists in signal transduction between Ha-ras and c-fos, as fos antisense has been shown to abrogate Ha-ras-mediated activation of other genes such as collagenase and transin (27, 28). Intriguingly, Ha-ras gene expression was also reduced in anti-fosRz-treated cells (data not shown). Finally, c-jun expression is also linked to Ha-ras-directed pathways, as the Ha-ras gene product potentiates c-jun activity by phosphorylating Jun (29). Collectively, these observations describe the existence of an intracellular network of cros-signaling involving transcriptional and posttranslational regulation. These pathways appear to be activated in response to a diverse array of stimuli, such as growth factors, tumor promoters, and cancer chemotherapeutic agents.

As mentioned earlier, the upstream regulatory sequences of the mdr-1 gene contain an AP-1 binding site (30). Even though this region is not the dominant promoter for mdr-1 in all circumstances, it has been shown to be required for full promoter activity in Chinese hamster ovary cells (18). In addition, the AP-1-containing promoter may be active in cell lines that overexpress mdr-1 RNA without gene amplification (31, 32), which is precisely the situation encountered in A2780AD cells. Therefore, downregulation of mdr-1 RNA after anti-fos ribozyme action is part of the cascade effecting reversal of the MDR phenotype.

Our results also indicate the efficacy of an anti-MDR ribozyme in reversing the MDR phenotype. This parallels the use of anti-MDR ribozymes to suppress mdr-1 mRNA in other model systems (33, 34). With extremely high levels of drug resistance, other mechanisms of resistance may be activated and may even predominate. It would be intriguing to use the anti-fos ribozyme in cell lines in which suppressing mdr-1 expression is insufficient to restore drug cytotoxicity or in which multidrug-resistant-related protein is overexpressed (7).

Interestingly, A2780ADpmdrRz cells also displayed diminished gene expression of c-fos, topoisomerase I, and p53 (Fig. 1). However, it must be noted that those experiments represent a one-time measurement, since the pH4 apr1 neo plasmid containing the anti-MDR ribozyme uses the β-actin promoter to drive constitutive expression of the ribozyme and is not inducer driven as shown with A2780AD fosRz cells. Therefore, the reduced gene expression observed may be less a result of direct effects on transcriptional regulation of these genes and more a reflection of selection pressures on a cell subline displaying the drug-sensitive phenotype and containing normal levels of the mdr-1 gene product.

Finally, the differential pattern of drug sensitivity between the two ribozymes may offer mechanistic explanations for action of the two genes in drug resistance. The observation that the anti-fos ribozyme reversed actinomycin D resistance more quickly may suggest that c-fos may modulate genes other than mdr-1 which also contribute to the MDR phenotype. One such candidate is topoisomerase I, also implicated in atypical MDR, in which mdr-1 gene expression is unperturbed (6). Our studies demonstrate that anti-fos ribozyme action was also resulted in reduced expression of topoisomerase I.

In conclusion, the data presented here demonstrate the efficacy of an anti-fos ribozyme in reversing the MDR phenotype, while reducing expression of mdr-1, c-jun, p53, and topoisomerase I. The anti-fos ribozyme was equally, if not more, effective than the anti-MDR ribozyme. This suggests the primacy of c-fos in many drug-resistance processes.

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