Selective Inhibition of P-glycoprotein Expression in Multidrug-Resistant Tumor Cells by a Designed Transcriptional Regulator

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ABSTRACT

Selective inhibition of the multidrug resistance 1 (MDR1) gene and its product, the P-glycoprotein, a membrane transporter responsible for multidrug resistance, could be an important approach for enhancing cancer therapeutics. An emerging strategy for selective gene regulation involves designed zinc finger proteins that can recognize specific sequences in the promoter regions of disease-related genes. Herein, we investigate the behavior of clones of multidrug-resistant NCI/ADR-RES breast carcinoma cells displaying ponasterone-inducible expression of a designed transcriptional repressor targeted to the MDR1 promoter. The controlled production of this novel repressor resulted in major reductions in P-glycoprotein levels in these otherwise highly drug-resistant tumor cells. The regulated reduction of MDR1 expression in NCI/ADR-RES cells was accompanied by a marked increase in the rate of uptake of the P-glycoprotein substrate rhodamine 123. In addition, the cytotoxicity profile of the antitumor drug doxorubicin was dramatically altered in the induced cells compared with controls. The expression levels of other genes were examined both by a DNA array analysis of approximately 2000 genes and by biochemical techniques. Although some changes were observed in mRNA levels of nontargeted genes, the most dramatic effect by far was on MDR1, indicating that the action of the designed transcriptional repressor was quite selective. This study suggests that designed transcriptional regulators can be used to strongly and selectively influence expression of cancer-related genes, even under circumstances of extensive amplification of the target gene.

The ability to selectively regulate the expression of genes associated with the development or progression of cancer would constitute a potentially powerful therapeutic strategy. Such an approach could be used to inhibit the expression of oncogenes or of genes involved in resistance to therapy; alternatively, it could be used to enhance the expression of tumor suppressor genes or their targets. Recent developments have indicated that this approach may indeed be feasible. Thus, combinatorial library strategies can be used to identify novel DNA-binding domains capable of selective recognition of the upstream regulatory regions of specific genes. When a selective DNA-binding domain is coupled to an appropriate trans-activating or repressor domain, a highly specific transcriptional regulator protein is created.

A particularly important and demanding aspect of the regulation of cancer-related genes concerns the phenomenon of resistance to multiple anticancer drugs due to overexpression of P-glycoprotein, the product of the MDR1 gene. The P-glycoprotein is a 170-kDa membrane ATPase that can expel many types of drug molecules from cells (Ling, 1997; Ambudkar et al., 1999). Moderate overexpression of P-glycoprotein is usually associated with increased levels of MDR1 transcription, whereas higher levels of overexpression are often associated with extensive gene amplification (Ambudkar et al., 1999). Although multidrug resistance can be modulated by pharmacological agents that interfere with P-glycoprotein function (Orlowski and Garrigos, 1999), selective inhibition of P-glycoprotein expression offers an alternative therapeutic strategy (Alahari et al., 1996; Wang et al., 1999). The MDR1 gene is regulated by the combined actions of several transcription factors that bind to its promoter region (Scotto and Johnson, 2001). In particular, an EGR1/SP1/WT1 site at positions −69 to −41 is functionally significant for control of MDR1 gene expression (McCoy et al., 1999) and thus represents a potential therapeutic target. We have addressed this target by designing and using a repressor that specifically binds adjacent to the EGR1/SP1/WT1 site in the MDR1 promoter.

The Cys2-His2 type of zinc finger (Zif) provides a particularly useful modular structure for the creation of novel DNA-binding proteins. To a first approximation, each C2H2 zinc finger domain of a multi-Zif transcription factor makes sequential contacts with bases within the major groove of DNA.

ABBREVIATIONS: MDR, multidrug resistance; Zif, zinc finger; Rh123, rhodamine 123; PBS, phosphate-buffered saline; R-PE, R-phycocerythrin; MHC, major histocompatibility complex.
Because of this modular nature, individual Zifs can be combined to form new DNA-binding proteins with novel sequence specificity (Beerli et al., 1998; Kim and Pabo, 1998). In addition, combinatorial laboratory techniques such as phage display (Choo and Klug, 1994; Rebar and Pabo, 1994; Wu et al., 1995), yeast one-hybrid selection (Cheng et al., 1997), and bacterial two-hybrid selection (Joung et al., 2000) have been used to identify novel Zifs with altered DNA recognition capabilities. When combined with transactivator or repressor domains to form novel transcriptional regulators, these zinc finger proteins have shown impressive effects in reporter gene assays (Kim and Pabo, 1997; Beerli et al., 2000; Kang and Kim, 2000), and in terms of binding affinities to naked DNA in vitro (Kim and Pabo, 1998; Moore et al., 2001). However, the accessibility and responsiveness of endogenous genes embedded within chromatin may be quite different from those of genes present on episomes (Kadonaga, 1998), such as are used in reporter gene assays (Kim and Pabo, 1997; Beerli et al., 1998; Kang and Kim, 2000), and in terms of binding affinities to naked DNA in vitro (Kim and Pabo, 1998; Moore et al., 2001). Therefore, the accessibility and responsiveness of endogenous genes may be a challenging problem.

We have previously described the use of a yeast combinatorial library approach to select novel zinc fingers able to recognize sequence motifs in the MDR1 promoter (Bartsevich and Juliano, 2000). The selected Zifs were combined with native Zifs derived from the SP1 or Zif 268 transcription factors to form a five zinc finger DNA-binding module capable of highly selective recognition of a 15-base sequence (GGG TGG GCT GAG) overlapping the EGR1/SP1/WT1 site of the MDR1 promoter. Five Zif modules were coupled to either the VP16 viral transactivation domain or to a mammalian KRAB-A repression domain (Witzgall et al., 1994). The five Zif chimeric proteins containing the VP16 or KRAB-A domains displayed, respectively, strong activation or strong repression of MDR1 promoter-reporter constructs in transient transfection assays in mammalian cells. A chimera comprised of five Zifs and two KRAB-A repressor domains termed K2-5F was selected for further study.

We have now gone on to examine the possibility of regulating P-glycoprotein expression and drug responses in multidrug-resistant human cancer cells that possess highly amplified MDR1 genes. The NCI/ADR-RES breast cancer cell line displays a high level of resistance to doxorubicin and to several other agents, due to MDR1 gene amplification and consequent massive overexpression of the P-glycoprotein (Fairchild et al., 1987; Scudiero et al., 1998). Herein, we have used a ponasterone-inducible system to provide regulated expression of the K2-5F repressor protein in stably transfected subclones of NCI/ADR-RES. We demonstrate that expression of K2-5F results in a dramatic reduction in the expression of P-glycoprotein. This is accompanied by increased uptake of rhodamine 123 (Rh123), a fluorophore that is a P-glycoprotein substrate, and by a marked increase in sensitivity to the toxic effects of doxorubicin. In addition, we have used DNA array analysis to evaluate the impact of K2-5F induction on the expression of nontarget genes. In summary, we have used a designed transcription factor to attain significant and selective regulation of a highly amplified gene that plays an important role in tumor progression.

**Materials and Methods**

**Cell Culture.** MCF7 and doxorubicin-resistant NCI/ADR-RES human breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). NCI/ADR-RES was formerly termed MCF7/ADR (Scudiero et al., 1998). The cells were cultured in minimal essential medium with 1-glutamine (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum and containing the antibiotics penicillin/streptomycin.

**Construction of Plasmids.** Plasmids pVgRXR, pIND, and pIND/lacZ were obtained from Invitrogen (Carlsbad, CA). Plasmid pVgRXR contains the coding sequences for the modified subunits of edysone receptor, RXR and VgEcR, under the control of constitutive Rous sarcoma virus and cytomegalovirus promoters. Plasmid pIND contains five copies of a modified edysone response element (E/GRE) upstream of a minimal promoter. Plasmids pVgRXR and pIND contain resistance genes to Zeocin or G418, respectively. Plasmid pIND/lacZ contains a lacZ gene placed into the multiple cloning site of pIND. An inducible version of the K2-5F repressor was created as follows. The polylinker in plasmid pIND was replaced by an Nhel-Pmel fragment from vector pcDNA3.1(−)/Myc-HisA (Stratagene, La Jolla, CA), resulting in vector pINDm that contains the polylinker from the pcDNA3 vector along with a Myc epitope and a polyhistidine tag. An 849-base pair Xbal-HindIII fragment from plasmid pc2-K2-5F (Bartsevich and Juliano, 2000) containing the entire coding sequence of K2-5F, flanked at the N terminus with a nuclear localization signal, was subcloned into vector pINDm, leading to plasmid pIND/K2-5F. This plasmid expresses a Myc- and polyhistidine-tagged version of K2-5F under the control of an edysone/ponasterone-inducible promoter. Plasmid pc2-K5F expresses K2-5F under the control of a constitutive cytomegalovirus promoter (Bartsevich and Juliano, 2000).

**Transient Transfection Experiments.** NCI/ADR-RES cells were transiently transfected with pc2-K5F using Superfect (QIAGEN, Valencia, CA) or were cotransfected with the following vectors: 1) pVgRXR and pIND/K2-5F or 2) pVgRXR and pIND. After a 15-h recovery, the transfected cells were induced by the addition of the edysone analog ponasterone A (dissolved in absolute ethanol) to the culture media to the final concentration of 5 μM. After 24-h induction, cells were subjected to further analysis.

**Stable Cell Line Production.** NCI/ADR-RES cells were first transfected with pVgRXR, which encodes the modified subunits of the edysone receptor, VgEcR and RXR. Forty-eight hours after transfection, cells resistant to Zeocin were selected in medium containing 0.5 mg/ml Zeocin (Invitrogen). Stable cell lines expressing the heterodimeric edysone receptor were selected by testing for ponasterone A-inducible expression of β-galactosidase activity after transfecting with the plasmid (pIND/lacZ). Receptor-expressing cell lines were then transfected with linearized inducible expression vector pIND/K2-5F and selected using 1 mg/ml G418 (Invitrogen). Clones were tested for ponasterone-inducible K2-5F expression by Western blot after a 24-h induction. Ten positive clones were selected and maintained in the presence of Zeocin (0.3 mg/ml) and G418 (0.5 mg/ml). Clones displaying ponasterone-inducible expression of K2-5F are designated as R1, R2, etc., with the “R” signifying the inducible repressor.

**Flow Cytometry.** For measurement of cell surface proteins, cells (1 × 10⁶) were trypsinized briefly, washed, and resuspended in 100 μl of cold isotonic phosphate-buffered saline (PBS). Cells were incubated on ice for 45 min with the appropriate primary antibody at 1:100 dilution for the detection of P-glycoprotein using anti-P-glycoprotein antibody MRK-16 (Kamiya Biochemicals, Thousand Oaks, CA); MHC using anti-HLA class I antibody (Accurate Chemical & Scientific, Westbury, NY); and integrin α2, or α3 subunits using anti-human integrin α subunit antibodies p166 or p1β5 (Invitrogen). After three washes with 10% serum/PBS, an R-phycocerythrin (R-PE)-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) was used as the second antibody at 1:100 dilution. After a 30-min
incubation on ice, cells were washed three times with 10% serum/ PBS and resuspended in 500 μl of PBS. The level of R-PE fluorescence was measured on a flow cytometer (BD Biosciences, San Jose, CA) as described previously (Alahari et al., 1996)

Western Blotting. Cells were lysed in modified radiolabeled precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet-40, 0.5% deoxycholate, 5 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, and 0.1% SDS), and lysates were centrifuged at 12,000 rpm for 10 min at 4°C. Equal amounts of protein (20 μg) were mixed with 2× SDS sample buffer and boiled for 5 min. The proteins were subjected to 15% (for detection of K2-5F) or 4 to 20% gradient (Invitrogen) (for detection of P-glycoprotein and β-actin) SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Expressed protein K2-5F was detected using monoclonal anti-c-myc antibody 9E10 (Berkley Antibody Company, Richmond, CA) at a dilution of 1:2000. P-glycoprotein was detected using monoclonal anti-P-glycoprotein antibody C219 (Signet Laboratories, Dedham, MA) at 1:500. β-Actin was detected by anti-actin antibody (Sigma-Aldrich) at a dilution of 1:6000. Secondary antibody was peroxidase-conjugated goat antimouse IgG antibody (Calbiochem, San Diego, CA) at a dilution of 1:5000. Signals were detected by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Piscataway, NJ). Membrane stripping was conducted in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.75% β-mercaptoethanol) at 65°C for 30 min. Stripped membranes were washed and blocked with 5% nonfat dry milk in 1× PBS before further Western blotting.

RNA Extraction and Northern Blotting. Total RNA was isolated using a RNA kit (QIAGEN). Northern blotting was done according to standard protocols (Alwine et al., 1977). The blot was hybridized with a 32P-labeled human MDR1 cDNA probe. The template for probe labeling was a gel purified reverse transcription-polymerase chain reaction product of human MDR1, with the for-plate for probe labeling was a gel purified reverse transcription-antibody C219 (Signet Laboratories, Dedham, MA) at 1:500. Expression of human K2-5F was detected using monoclonal anti-c-myc antibody 9E10 (Berkley Antibody Company, Richmond, CA) at a dilution of 1:2000. P-glycoprotein was detected using monoclonal anti-P-glycoprotein antibody C219 (Signet Laboratories, Dedham, MA) at 1:500. β-Actin was detected by anti-actin antibody (Sigma-Aldrich) at a dilution of 1:6000. Secondary antibody was peroxidase-conjugated goat antimouse IgG antibody (Calbiochem, San Diego, CA) at a dilution of 1:5000. Signals were detected by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Piscataway, NJ). Membrane stripping was conducted in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.75% β-mercaptoethanol) at 65°C for 30 min. Stripped membranes were washed and blocked with 5% nonfat dry milk in 1× PBS before further Western blotting.

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DNA Array Analysis. The effects of the induction of the K2-5F repressor protein on expression of a broad spectrum of genes was evaluated using DNA array technology. Total RNA was isolated using a kit (QIAGEN). Complementary DNA was synthesized from 0.7 μg of isolated total RNA with a T7- or T3-primed cDNA kit (Invitrogen). The BioArray High Yield RNA Transcription kit (Affymetrix, Santa Clara, CA) was used to produce biotin-labeled cRNA. Fifteen micrograms of fragmented cRNA (incubated at 94°C for 35 min in 5× fragmentation buffer: 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, and 150 mM MgOAc) was added to a hybridization cocktail. Human Cancer Chip Arrays (2059 genes) from Affymetrix, Santa Clara, CA) were hybridized sequentially with plasmids pVgRXR and pIND/K2-5F, and pVgRXR and empty vector pIND, and induced with ponasterone A for 24 h. As seen in Fig. 1a, K2-5F was readily visualized by Western blotting in cells transfected with pCK2-5F, and in ponasterone-induced cells transfected with pVgRXR and pIND/K2-5F, but not in cells expressing the pIND empty vector. The effect of transient transfection following by induction of K2-5F on cell surface levels of P-glycoprotein was examined by immunostaining and flow cytometry. A cell population expressing ponasterone-induced K2-5F showed a substantial left shift of the P-glycoprotein profile (Fig. 1b), whereas ponasterone-treated untransfected cells were unaffected. Because only approximately 20% of the cells were transfected in these studies, the significant left shift in the population profile suggested that K2-5F was having a strong effect on P-glycoprotein levels in the minority of cells where it was being expressed.

Inducible Expression of K2-5F in Stable Transfectants of NCI/ADR-RES Cells. NCI/ADR-RES cells were transfected sequentially with plasmids pVgRXR and pIND/K2-5F followed by selection with Zeocin and G418, respectively. Forty clones resistant to both antibiotics were selected, and 10 of these showed ponasterone-induced expression of the repressor K2-5F at various levels. Figure 2 shows the induced K2-5F in some of these clones, as detected by Western blot. The expression of K2-5F seemed to be tightly controlled by the inducer. The expression of K2-5F was dose-dependent with an optimum reached at approximately 15 μM ponasterone (data not shown); it was also time-dependent and increased progressively over a period of several days.

Inhibition of Cell Surface Expression of P-glycoprotein Measured by Flow Cytometry. The effect of ponasterone induction of K2-5F on cell surface levels of P-glycoprotein was examined in several independent subclones of NCI/ADR-RES transfected using immunostaining and flow cytometry. As illustrated in Fig. 3 for the subclones R-8 (a–c) and R-5 (d–f), there was a progressive reduction in P-glyco-
The membrane was probed with monoclonal anti-c-myc antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody. The membrane was developed using Enhanced Chemiluminescence (ECL) detection technology. Three independent experiments were performed, each including subclones R-5 and R-8, and the parental cell line (in other words, there should be no nonspecific effect of ponasterone itself on the gene). 3) The hybridization intensity must be above 100 arbitrary units to ensure a high signal-to-noise ratio. As shown in Table 1, of 2059 genes surveyed, eight met these criteria. As anticipated, MDR1 displayed the most specific effect of ponasterone on the gene transcription. In addition, the effects of K2-5F induction were fully reversible. Thus, as seen in Fig. 4, after 3 days of ponasterone treatment of NCI/ADR-RES cells had caused a major reduction in cell surface expression of P-glycoprotein, removal of the ponasterone allowed the expression levels to return almost to control levels over a 3-day recovery period.

**Effects on P-glycoprotein Levels Measured by Western Blot and on MDR1 mRNA Measured by Northern Blot.** The effect of the repressor K2-5F on the expression levels of P-glycoprotein was also evaluated by Western blotting. As seen in Fig. 5a (top), after 4 days of ponasterone treatment there was a marked reduction in total cellular levels of P-glycoprotein in subclones R-5, R-8, and R-40. As shown in Fig. 5a (bottom), ponasterone treatment had no effect on levels of actin expression. This suggests, again, that the action of K2-5F is selective for the MDR1 gene. No change of P-glycoprotein levels was observed in untransfected NCI/ADR-RES cells in response to ponasterone treatment. As seen in Fig. 5b, a similar pattern was observed when levels of MDR1 message were evaluated by Northern blotting. Thus, MDR1 message levels showed a marked reduction in clones R-5 and R-8 in response to ponasterone treatment, whereas levels of actin message were unaffected.

**Array Analysis.** To further evaluate the selectivity of K2-5F in regulating gene expression, we used DNA microarray technology. Three independent experiments were performed, each including subclones R-5 and R-8, and the parental cell line NCI/ADR-RES. Because our preliminary experiments suggested that K2-5F induction would have a large effect on MDR1 gene expression, we limited our analysis to genes that also showed a strong response. Thus, only genes that met the following rather stringent criteria were considered to have shown a significant response. 1) After a 4-day induction of K2-5F using ponasterone, there must be at least a 3-fold change in mRNA level in both stable cell lines; this must occur in all three of the independent experiments. 2) The mRNA level must not show significant change in the parental cell line (in other words, there should be no nonspecific effect of ponasterone itself on the gene). 3) The hybridization intensity must be above 100 arbitrary units to ensure a high signal-to-noise ratio. As shown in Table 1, of 2059 genes surveyed, eight met these criteria. As anticipated, MDR1 displayed the most significant change (approximately a 15-fold reduction). The genes for human metastasis-associated mta1 (GenBank U35113), CaN19 (GenBank M87068), and growth-arrest-specific protein (GenBank L13720) also had significantly reduced message levels. Examination of the promoter regions of these genes using the Vector NTI analysis program failed to reveal any sites with significant consensus with the K2-5F
target site in MDR1. Four genes showed significant increases in message levels subsequent to K2-5F induction. These were human mitogen-activated protein kinase kinase 3b (GenBank D87116), integrin α3 (GenBank M59911), semaphorin E (GenBank AB000220), and Bcl-Xl (GenBank Z23115). Interestingly, GC-rich regions were found within 500 base pairs of the transcription start sites for the four genes that showed increased message levels. None of these sites afforded a close match with the K2-5F target site, but there was some degree of homology. It is difficult to envision, however, how a strong repressor such as K2-5F would cause transcriptional activation by direct binding to one of these sites. If less stringent criteria were used, for example, a 2-fold change in mRNA levels, many more genes would need to be considered. However, many of the genes that displayed 1.5- to 2-fold changes in response to K2-5F induction did so consistently, with variation from one experiment to the next, and were thus not analyzed further.

**Effects on Uptake of a P-glycoprotein Substrate.** Rh123, a fluorescent dye, is a substrate for the P-glycoprotein and is commonly used to evaluate its drug-pumping activity via a flow cytometric assay (Twentyman et al., 1994). We compared Rh123 accumulation in drug-sensitive MCF7 cells, in the drug-resistant NCI/ADR-RES parental line, and in the R-8 and R-5 clones of NCI/ADR-RES that express the K2-5F repressor in inducible form. As seen in Fig. 6, a–c, the MCF7 cells rapidly accumulated Rh123, whereas the NCI/ADR-RES cells showed very little uptake; the uptake processes in these cells were unaffected by ponasterone treatment. In the cases of R-8 and R-5 (Fig. 6, a and b) Rh123 uptake was dramatically altered upon induction with ponasterone; the uninduced cells displayed uptake kinetics similar to that of

![Figure 3](https://example.com/figure3)
the parental NCI/ADR-RES cells, whereas the induced cells displayed rates of Rh123 accumulation intermediate between MCF7 and MCF/ADR. Thus, the reduced level of P-glycoprotein expression observed upon induction of the K2-5F repressor is reflected by an increased rate of accumulation of a P-glycoprotein substrate.

**Effects on Cytotoxicity of Doxorubicin.** We established dose-response curves for the effect of the anticancer drug doxorubicin on cell growth for the MCF7 and NCI/ADR-RES cell lines; the latter was found to be approximately 250-fold more resistant to doxorubicin than the former, in agreement with values in the literature (Batist et al., 1986; Mimnaugh et al., 1989). Furthermore, as seen in Table 2, exposure of the inducible subclones to verapamil, an inhibitor

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Stable Clones</th>
<th>Parental NCI/ADR-RES</th>
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<tbody>
<tr>
<td>Human MDR1</td>
<td>-15.5</td>
<td>+1.3</td>
</tr>
<tr>
<td>Human metastasis-associated mta1</td>
<td>-6.2</td>
<td>+1.7</td>
</tr>
<tr>
<td>Human CaN19</td>
<td>-3.6</td>
<td>+1.4</td>
</tr>
<tr>
<td>Homo sapiens growth-arrest-specific protein</td>
<td>+3.8</td>
<td>-1</td>
</tr>
<tr>
<td>Human mitogen-activated protein-kinase 3b</td>
<td>+3.7</td>
<td>-1.1</td>
</tr>
<tr>
<td>Homo sapiens semaphorin E</td>
<td>+3.1</td>
<td>-1.1</td>
</tr>
<tr>
<td>Homo sapiens bcl-xL</td>
<td>+3</td>
<td>-1.3</td>
</tr>
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\( P < 0.05 \) for the change versus control.

**TABLE 2**

Sensitization of subclones R-5 and R-8 to doxorubicin by verapamil

Subclones R-8 and R-5 were exposed for 24 h to various concentrations of doxorubicin in the presence or absence of 6.6 \( \mu M \) verapamil (added 5 min before doxorubicin). After a further 48-h period in complete growth medium, cell numbers were determined and expressed as percentage of control. An IC_{50} was obtained from the plot of cell number versus doxorubicin concentration.

<table>
<thead>
<tr>
<th>Clone</th>
<th>IC_{50} Verapamil</th>
<th>IC_{50} + Verapamil</th>
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<tbody>
<tr>
<td>R-5</td>
<td>21 ( \mu M )</td>
<td>2.1</td>
</tr>
<tr>
<td>R-8</td>
<td>20.3 ( \mu M )</td>
<td>2.6</td>
</tr>
</tbody>
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of the P-glycoprotein (Ambudkar et al., 1999), resulted in a substantial reduction in their resistance to doxorubicin. This indicates that the drug resistance profile of the subclones is due primarily to their expression of the P-glycoprotein. We then examined the effect of ponasterone on doxorubicin cytotoxicity in the inducible R-8 and R-40 clones. As seen in Fig. 7, a–c, the uninduced R-40 and R-8 cells displayed doxorubicin dose-response profiles similar to that of the NCI/ADR-RES parental cells, with an IC_{50} values of approximately 9 to 20 μM. Subsequent to ponasterone treatment, the dose-response profiles of the R-40 and R-8 clones were significantly left shifted, corresponding to IC_{50} values of 1.5 and 0.7 μM, respectively. Thus, induction of the K2-5F repressor led to a substantial reversal of doxorubicin resistance in these cells. Ponasterone treatment had no effect on the doxorubicin dose-response profile of the parental NCI/ADR-RES cells.

Discussion

In this study, we have examined the effect of K2-5F, a designed transcriptional repressor, on the expression and function of the MDR1 gene in highly drug-resistant NCI/ADR-RES carcinoma cells. Using an ecdysone/ponasterone responsive system, we found that regulated expression of K2-5F had robust effects on levels of MDR1 message and of its protein product, the P-glycoprotein multidrug transporter. Consequent to reductions in P-glycoprotein levels, the rates of drug uptake and the cytotoxic effects of an antitumor drug were substantially increased.

Overexpression of the P-glycoprotein leading to multidrug resistance has been associated with both amplification of the MDR1 gene and increased levels of transcription (Ambudkar et al., 1999). Recently, the promoter region of MDR1 has been carefully mapped (Scotto and Johnson, 2001) and a number of positive and negative regulators identified (Ogretmen and Safa, 1999; Scotto and Johnson, 2001). Clearly, the SP1/ERG1/WT1 site targeted by K2-5F is a key regulatory region for MDR1 transcription (McCoy et al., 1999), suggesting that it is an appropriate target for interdicting multidrug resistance. Thus, we have attained a very significant reduction in P-glycoprotein expression and a substantial reversal of drug resistance using a repressor directed to the SP1/ERG1/WT1 site. Other approaches to blocking MDR1 expression include antisense oligonucleotides and ribozymes, but these strategies have been problematic. The attainment of substantial reductions of P-glycoprotein levels using antisense has proven very difficult (Alahari et al., 1996, 1998). Although in some cases very impressive results have been obtained using ribozymes directed at MDR1 message (Wang et al., 1999), other studies have raised concerns about this approach (No author listed, 1997). In any case, our use of a designed transcriptional repressor seems to be a very effective strategy for MDR1 regulation.

Conceivably, it would seem possible to selectively block gene expression either by interfering with the binding of positively acting transcriptional regulators to the promoter being addressed, or by targeting transcriptional repressors to that promoter. However, our previous experience (Bartsevich and Juliano, 2000) and that of others (Fredericks et al., 2000) suggests that use of an active repressor domain is more likely to result in robust inhibition of message expression. In the present study, we have used two copies of a mammalian KRAB-A repressor domain as part of the K2-5F protein. KRAB repressor domains are believed to function by recruiting the corepressor KAP-1/TIF1/KRIP-1 (Kim et al., 1996; Abrink et al., 2001); KAP-1 then serves to recruit the NuRD complex that deacetylates histones and induces chromosomal condensation (Schultz et al., 2001), resulting in silencing of transcriptional activity. The effectiveness of the K2-5F repressor in inhibiting MDR1 transcription might be somewhat surprising, because KRAB repressors have been reported to function most effectively in the context of TATA-box containing promoters (Pengue and Lania, 1996), whereas MDR1 lacks such a motif (Scotto and Johnson, 2001). However, other articles suggest that KRAB domains effectively block transactivation driven by SP-1 (Licht et al., 1993), and thus the presence of an SP1/ERG1/WT1 site in MDR1 may contribute to the robust action of K2-5F.

The action of K2-5F in repressing the MDR1 gene seems to be quite selective. Clearly, the five Zifs of K2-5F display high selectivity for a 15-base pair sequence in the human MDR1 promoter (Bartsevich and Juliano, 2000). It can be calculated that precise recognition of 16 bases is sufficient to provide unique sequence specificity for the approximately 3.2 billion-base pair human genome. However, because only approximately one-third of the genome is comprised of genes (Lander et al., 2001), recognition of 15 bases is likely to provide a substantial degree of selectivity in terms of regulation of message levels. This is consistent with the lack of effect of K2-5F on expression of the several nontarget genes examined herein, including MHC antigen, actin, and several integrin subunits. In particular, because the MHC antigen promoter...

Fig. 7. Doxorubicin (Adriamycin) toxicity in response to K2-5F induction. Subclones R-8 (a) and R-5 (b), as well as the NCI/ADR-RES parental cell line (c), were treated with 15 μM ponasterone A (+) or solvent control (−) for 4 days. Uninduced (solid circles) and induced (open circles) cells were exposed for 24 h to various concentrations of Adriamycin. After a further 48-h period in complete growth medium, cell numbers were determined and expressed as percentage of control.
contains SP-1 sites (Gobin et al., 1999), as does the MDR1 promoter, the lack of effect on MHC expression supports the selective nature of the interaction of K2-5F with its MDR1 target. An even more stringent test of selectivity is provided by DNA array analysis. Subsequent to K2-5F induction only 8 of 2000 plus genes on the array displayed changes in message levels that we deemed significant. Furthermore, the change in MDR1 message levels (15-fold) was far greater than for any other gene. In addition, none of the genes that displayed changes in message levels seemed to have a promoter that would be a direct target of K2-5F; most likely, the observed changes were due to indirect effects. Thus, the K2-5F designed repressor seems to act on the MDR1 promoter with substantial, although not complete, specificity. Interestingly, recent studies using DNA array technology to evaluate effects caused by antisense oligonucleotides revealed much more diverse changes in nontarget message levels than those observed herein (Cho et al., 2001; Astrab-Fisher et al., 2002); this may suggest that designed transcription factors inherently possess greater selectivity than antisense molecules.

Highly drug-resistant lines such as NCI/ADR-RES massively overexpress P-glycoprotein. This is primarily due to extensive gene amplification; however, alterations in transcription regulation have also been observed in these cells (Ogretmen and Safa, 1999). It also seems possible that the multiple recombinational events leading to extensive gene amplification might, in some cases, alter the organization of the promoter regions of the amplified modules. This combination of possibilities makes multidrug resistance an extremely challenging problem for the application of designed transcription factors to gene regulation. However, because we observe reductions in MDR1 expression of 90% or more, this suggests that most or all copies of the amplified gene in NCI/ADR-RES cells can be regulated by K2-5F.

Previous studies have targeted repressor domains to the promoter of the Erb family of tyrosine kinases (Beerli et al., 2000) and to the DNA-binding sites of the PAX3 transcription factor (Fredericks et al., 2002); this may suggest that designed transcription factors inherently possess greater selectivity than antisense molecules.

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polymerase II promoters is influenced by the arrangement of basal promoter elements. *Proc Natl Acad Sci USA* **92**:1015–1020.


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