Inhibition of ABCB1 (MDR1) and ABCB4 (MDR3) expression by small interfering RNA and reversal of paclitaxel resistance in human ovarian cancer cells

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Introduction
Ovarian cancer is currently the leading cause of death from gynecologic malignancies in the United States. Paclitaxel, originally isolated from Taxus brevifolia (the Pacific yew), is a microtubule stabilizing chemotherapeutic drug used in the treatment of ovarian, breast, and nonsmall cell lung cancers (1, 2). The primary limitation of efficacy for paclitaxel is the development of drug-resistant cancer cells that exhibit the multidrug resistance phenotype (3). Overexpression of the ATP binding cassette transporters such as ABCB1 (MDR1) has been directly implicated in resistance to a broad spectrum of chemotherapeutic agents in vitro including paclitaxel, the anthracyclines, and the Vinca alkaloids (4-6). For various types of cancer, accumulated evidence from in vitro studies indicates that ABCB1 overexpression may be the predominant factor in limiting the efficacy of chemotherapeutic agents. Introduction and overexpression of the ABCB1 gene into drug-sensitive cells or into mice to produce transgenic animals confers resistance to the agents described above (7, 8). The ABCB1 gene encodes a membrane-bound P-glycoprotein (P-gp), which has been shown to transport a variety of chemotherapeutic drugs out of the cell in an energy-dependent process. Further study showed that some of these resistant cell lines also overexpress another ATP binding cassette transporter gene, ABCB4 (also known as mouse MDR2 or human MDR3; refs. 8, 9). ABCB4 expression has also been negatively correlated with clinical outcome (10). In brief, cell culture, animal, and clinical studies strongly suggest that overexpression of the ABCB1 gene may be the single most important determinant of drug resistance in some cancers.

Current strategies to prevent or reverse multidrug resistance have focused primarily on the development of agents that are competitive inhibitors of P-gp. Development of these agents for clinical use has been hindered by toxicity and limited efficacy (11). In addition, some studies have indicated that these and related P-gp inhibitors stimulate ABCB1 expression (12, 13). Alternative strategies involving the inhibition of transporter expression may offer superior mechanisms for reversing the multidrug resistance phenotype.

Small interfering RNAs (siRNAs) are double-stranded RNA molecules that induce sequence-specific degradation of homologous single-stranded RNA. In plants and insects, siRNA activity plays a role in host cell protection against viruses and transposons (14, 15). From a biological research perspective, siRNA is proving to be a very powerful technique to “knockdown” specific genes, thereby enabling the evaluation of their physiologic roles in Caenorhabditis elegans, Drosophila melanogaster, and human cells. siRNA
technology has several major advantages over other post-transcriptional gene silencing techniques (such as antisense or gene knockout technology), in that it is easier to deliver, requires only small doses of siRNA to produce its silencing effect, and can inactivate a gene at almost any stage in development.

In this study, the utility of siRNA to reverse paclitaxel resistance in human ovarian cancer cells selected for resistance by treatment with paclitaxel is evaluated. These cells have been characterized previously and exhibit the classic multidrug resistance phenotype accompanied by increased expression of ABCB1 (16). In this investigation, some of these resistant cell lines were also found to overexpress ABCB4. The purpose of this study was to characterize siRNA inhibition of ABCB1 and ABCB4 in paclitaxel-resistant ovarian cancer cells and to determine if siRNA inhibition might offer advantages over classic P-gp inhibitors.

Materials and Methods

Cell Culture

The human ovarian cancer cell line SKOV-3 and its paclitaxel-resistant subline SKOV-3TR were established as reported previously (16, 17). The MCF-7TR breast cancer cell line and OVCAR8TR ovarian cancer cell line were established using a similar protocol. These cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The SKOV-3TR, OVCAR8TR, and MCF-7TR-resistant cell lines were grown in medium containing 0.3, 0.2, and 0.03 μmol/L concentrations of paclitaxel, respectively.

Synthesis of ABCB1 and ABCB4 siRNA

The siRNA sequence targeting ABCB1 (Genbank accession no. NM_000927) or ABCB4 (Genbank accession no. NM_018849) corresponded to coding regions of these genes. Two target sequences were selected for each gene: ABCB1a, ABCB1b, ABCB4a, and ABCB4b (Table 1). Each of the 19-nucleotide siRNAs contained a 3'-dTdT extension. Synthetic siRNA molecules, as described in Table 1, were obtained from Qiagen (Chatsworth, CA). The siRNAs were dissolved by adding 1 mL of the buffer [100 mmol/L potassium acetate, 30 mmol/L HEPES-KOH, and 2 mmol/L magnesium acetate (pH 7.4)] to each tube, heated for 1 minute at 90°C and 60 minutes at 37°C, and kept at −20°C until the following transfection experiment.

Vector-Based ABCB1 and ABCB4 siRNA

Currently, siRNA can be introduced by transfection of short synthetic siRNA or via expression from an appropriate vector as a small RNA hairpin (siRNAs with a self-complementary stem loop). The plasmid pSuppressorNeo was purchased from Imgenex Corp. (San Diego, CA). These vectors employ the type III class RNA polymerase promoter U6 to drive the expression of siRNA molecules. The pSuppressorNeo vector DNA (provided in the kit) was digested with SalI and XhoI to generate compatible ends for cloning. To add compatible restriction sites, the XhoI site was placed at the 5’ end and a XhoI site was added to the 3’ end of the primer sequences. The XhoI restriction site is compatible with the SalI site and will allow cloning into a SalI site (see manufacturer’s protocol for details). Plasmids expressing ABCB1 or ABCB4 siRNA hairpins were constructed by cloning the ABCB1 or ABCB4 coding region into pSuppressorNeo according to the manufacturer’s instructions. The primers used to generate the human ABCB1 and ABCB4 specific siRNAs were obtained from Qiagen. For example, to generate the ABCB1 siRNA expression plasmid, a double-stranded oligonucleotide siRNA insert was created by annealing primers corresponding to nucleotides 889 to 908 of the ABCB1 cDNA coding region with a six-nucleotide spacer (AGTACT). This insert was cloned to pSuppressorNeo plasmid. Two target sequences for each gene were selected for testing as described above. To compare the efficiency, we used the same sequence (Table 1) for vector-based siRNA and synthetic siRNA.

Synthetic siRNA Transfection

SKOV-3TR and OVCAR8TR were cultured as described above. For transfection, cells were either plated on 96-well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays or plated on dishes for Northern blot RNA isolation or Western blot protein isolation. Transfections were performed with TransMessenger transfection reagent (Qiagen) as directed by the manufacturer. For ABCB1 and ABCB4, each 96-well plate received 0.1 μg siRNA per well in a volume of 200 μL in triplicate, and each 60 mm dish received 5 μg siRNA per dish in a volume of 10 mL. Medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum 24 hours after transfection. Total RNA was isolated after 48 hours of siRNA transfection.

Table 1. Synthetic siRNAs used in this study

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>siRNA</th>
<th>siRNA (Sense Strand Only) 5’ to 3’</th>
<th>Target Location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>ABCB1a</td>
<td>r(UGCGACAGGAGAUAGGCUCU)TT</td>
<td>889</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ABCB1b</td>
<td>r(GCGAAGCAGUGGUUCAGGU)TT</td>
<td>2,113</td>
</tr>
<tr>
<td>ABCB4</td>
<td>ABCB4a</td>
<td>r(CUCAUAUCCGGCCUAACAGU)TT</td>
<td>2,387</td>
</tr>
<tr>
<td>ABCB4</td>
<td>ABCB4b</td>
<td>r(GAGGCCAACGGCCUAUGAGU)TT</td>
<td>13,392</td>
</tr>
</tbody>
</table>
Transfection of Plasmids Expressing siRNA into Resistant Cells

SKOV-3TR and OVCAR8TR were grown to 40% to 70% confluence in complete medium. Using Lipofectamine Plus transfection reagent (Invitrogen, Carlsbad, CA) and the suggested method, the cells were transfected with pSuppressorNeo plasmids expressing siRNA ABCB1, ABCB4, or just pSuppressorNeo empty vector without insert as a control. Medium containing 250 μg/mL G418 (Invitrogen) was added to the cells 24 hours after transfection. Stable G418-resistant clones were selected after 8 to 10 days of transfection for MTT cytotoxicity assay or Northern blot analysis.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted using Trizol reagent (Invitrogen) and quantified by a spectrophotometer. Total RNA (6 μg) was treated with formaldehyde and subjected to electrophoresis in a 1.2% agarose gel using standard techniques. The gels were transferred to Hybond nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) and probed with [32P]-labeled ABCB1 or ABCB4 specific gene probes that had been labeled using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). The isolation and characterization of ABCB1 and ABCB4 cDNA specific probes were used as described previously (18-20). The sequence of cDNA fragments encoding part of the transmembrane segment is sufficiently divergent to discriminate between these two genes (18). The full-length cDNA of the ABCB1 plasmid (pGEM3ZF (-)XBA-MDR1.1) and ABCB4 plasmid (p3Omega-MDR3) were purchased from American Type Culture Collection (Manassas, VA). The ABCB1 specific gene probe was prepared by purifying a 600-bp SstI-KpnI fragment from the ABCB1 plasmid, and the ABCB4 specific gene probe was prepared by purification of a 450-bp HindII-FspI fragment from the ABCB4 plasmid as described previously (18).

SDS-PAGE and Western Blot Analysis

Western blots were performed using an Enhanced Chemiluminescence Plus Western Blotting Detection Kit (Amersham Pharmacia Biotech). Eighty-percent confluent cells were washed with PBS and detached with trypsin-EDTA. Equal numbers of paclitaxel-sensitive or paclitaxel-resistant cells transfected with ABCB1 siRNA were lysed in M-PER Mammalian Protein Extraction Reagent buffer (Pierce Chemical Co., Rockford, IL) and centrifuged at 1,200 × g for 15 minutes at 4°C to remove insoluble debris. The detergent soluble fraction was loaded in equal aliquots of protein and resolved using 6% SDS-PAGE. The transferred nitrocellulose blot was blocked with 5% fat-free milk powder in TBS-T (pH 7.6; 20 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.01% Tween 20) at room temperature for 2 hours. The membrane was immunoblotted with monoclonal antibody C219 (product ID SIG-8710, Signet, Dedham, MA) for human ABCB1 and polyclonal antibody H-300 (Santa Cruz Biotechnology, Santa Cruz, CA) for human β-actin in TBS-T for 2 hours at room temperature. The molecular weight of ABCB1 encoded P-gp is 170 kDa, while ABCB4 encoded P-gp is 140 kDa. Antibody C219 recognized both of the ABCB1 and ABCB4 proteins (21, 22). Detection was performed using the reagents provided in the Enhanced Chemiluminescence Plus kit.

Cytotoxicity Assay

In vitro cytotoxicity assays were performed by MTT assays as described previously (23). MTT was obtained from Sigma Chemical Co. (St. Louis, MO). Briefly, for synthetic siRNA transfection, 2 × 10^3 cells per well were plated in 96-well plates with RPMI 1640 containing ABCB1 or ABCB4 siRNA (0.1 μg per well in volume of 200 μL) with increasing concentrations of paclitaxel from 0.0001 to 1 μmol/L. For vector-based siRNA transfection, 2 × 10^3 cells of stable ABCB1 or ABCB4 transfected clones were plated in 96-well plates with RPMI 1640 containing the same increasing concentrations of paclitaxel. Untransfected SKOV-3 and OVCAR8 were used as controls. After culturing for 6 days, 10 μL of MTT (5 mg/mL in PBS) was added to each well and incubated for 4 hours. After dissolving the resulting formazan product with acid isopropanol, the absorbance was read on a BT 2000 Micro-kinetics Reader (Bio-Tek Instruments, Inc., Winooski, VT) at a wavelength of 490 nm. The IC_{50} is defined as the drug concentration required to reduce the A_{490} nm to 50% of the control value. Fold reversal is the IC_{50} for paclitaxel-resistant cells divided by the IC_{50} for the siRNA-treated paclitaxel-resistant cells. The percentage of cell growth was calculated by defining the absorbance of parental cells not treated with paclitaxel as 1.0 and the value of the no-cell control to 0. Experiments were performed in triplicate. MTT cytotoxicity assays were also performed with 5 μmol/L verapamil (Sigma Chemical) using the same protocol as above. Presented results reflect at least three independent experiments performed in triplicate.

Results

ABCB1 and ABCB4 Overexpression in Paclitaxel-Resistant Cells

The paclitaxel-resistant SKOV-3TR, OVCAR8TR, and MCF-7TR ovarian cancer cell lines were evaluated for expression of ABCB1 and ABCB4 by Northern analysis. ABCB1 was found to be overexpressed in all three resistant cell lines, while ABCB4 showed modest overexpression in SKOV-3TR and more significant overexpression in OVCAR8TR and MCF-7TR. The expression of ABCB1 and ABCB4 in SKOV-3TR and OVCAR8TR is shown in Figs. 1 and 3, with MCF-7TR data not shown.

siRNA Inhibition of ABCB1 and ABCB4 mRNA Expression in Paclitaxel-Resistant Cells

To determine whether siRNA specific to the ABCB1 or ABCB4 gene sequences could inhibit gene expression, we first tested the synthetic siRNA and vector-based siRNA. Two different siRNAs were synthesized per gene: ABCB1a, ABCB1b, ABCB4a, and ABCB4b (see Materials and Methods for details). In addition, with the pSuppressorNeo system, two ABCB1 and two ABCB4 siRNA expression plasmids were created using the same sequences as defined for the
synthetic siRNA, mRNA expression of ABCB1 and ABCB4 was evaluated by Northern blot analysis. The results showed that the expression of ABCB1 was significantly decreased by addition of either the synthetic or the vector-based siRNA, although the vector-based method was less effective than the synthetic siRNA (Figs. 1 and 2). Both the SKOV-3TR and the OVCAR8TR cell lines revealed similar results. Targeting ABCB4 with specific synthetic or vector-based siRNAs also decreased ABCB4 mRNA to a similar magnitude (Fig. 3).

**siRNA Inhibition of ABCB1 Protein Expression in Paclitaxel-Resistant Cells**

The ABCB1 protein expression in siRNA transfected SKOV-3TR and OVCAR8TR cells was evaluated by Western blot analysis. As shown in Fig. 4, levels of ABCB1 protein were decreased 48 hours after transfection with the synthetic siRNA. The levels of ABCB1 proteins were also decreased after transfection with vector-based siRNA but less effectively than with the synthetic siRNA (data not shown).

**Paclitaxel Resistance in ABCB1 or ABCB4 Transfected Cells**

The ABCB1 or ABCB4 siRNA-mediated reversal of the paclitaxel-resistant phenotype was evaluated by comparison of the IC₅₀ values determined by MTT in siRNA-treated resistant and control cell lines. Cytotoxicity was measured 6 days after treatment with siRNA. As measured by MTT assay, the IC₅₀ values of the siRNA treated SKOV-3TR and OVCAR8TR cells were lower as compared with the untreated resistant lines, suggesting that ABCB1 siRNA inhibits ABCB1 expression and partially restores the sensitivity of these resistant cell lines to paclitaxel (Table 2). For ABCB1, the inhibition by synthetic siRNA was more effective than that by the vector-based siRNA. Cell lines treated with the synthetic siRNA of ABCB1 demonstrated 7- to 12.4-fold reduction in paclitaxel resistance as compared with 4.7- to 7.3-fold reduction of resistance by transfection with vectors containing the ABCB1 sequence. In contrast, the ABCB4 siRNA duplex with resultant decrease in ABCB4 RNA expression resulted in only modest reduction of paclitaxel resistance (Table 2).

**Comparison of Paclitaxel Resistance Reversal by Verapamil and ABCB1 siRNA**

The paclitaxel-resistant phenotype can be reversed by the calcium channel blocker verapamil, a reagent that has been used to evaluate cells for a functioning ABCB1.
efflux pump. To determine whether the activity of ABCC1 siRNA is as effective as verapamil, we compared SKOV-3TR exposed to synthetic ABCC1 siRNA to or to verapamil. ABCC1 siRNA-induced reversal of paclitaxel resistance was not as strong as that seen with verapamil (Table 2).

Discussion
In this study, it is demonstrated that both synthetic and vector-based expression of siRNA can specifically reduce the expression of the ABCC1 and ABCC4 genes, as well as P-gp expression for ABCC1, in paclitaxel-resistant cells. The effectiveness of siRNA in reducing expression of ABCC1 and ABCC4 was observed not only in paclitaxel-resistant SKOV-3TR cells but also in OVCAR8TR cells. Two approaches were taken to test whether siRNA was able to inhibit the expression of ABCC1 and ABCC4. Both synthetic and vector-based siRNAs significantly decreased the expression of ABCC1, although inhibition of ABCC1 by synthetic siRNA in paclitaxel-resistant cells was more effective than inhibition by the hairpin siRNA expressed from a vector containing the U6 promoter. Several reasons may explain this discrepancy, including transfection efficiency or the level of siRNA expression. We speculate that the amount of siRNA expressed from the vector is lower than the synthetic siRNA successfully transfected. Although, theoretically, siRNA is constantly expressed from the vector, the amount of siRNA expressed from these plasmids is not easily controlled, and current technology does not accurately monitor the siRNA level inside the cells. Further study using an inducible vector system to express siRNA may improve the efficiency of vector-based siRNA gene silencing.

By comparison, ABCC1 siRNA demonstrates a more potent reversal of paclitaxel resistance in both SKOV-3TR and OVCAR8TR as compared with ABCC4 siRNA. The ABCC4 gene is located on chromosome 7q21.1, 34 kb downstream of the ABCC1 gene (24). The ABCC1 and ABCC4 genes are very similar. They both contain 27 introns and are inserted at identical positions in the coding sequence (24). The proteins encoded by these genes have virtually identical hydropathy plots; they are 77% identical and 82% similar in amino acid sequence (18). The ABCC4 P-gp is normally involved in the transport of phospholipids from liver hepatocytes into bile but is also involved in the transport of paclitaxel and vinblastine, albeit inefficiently unless it is mutated (25). ABCC4 is also inhibited by verapamil, a classic inhibitor of the ABCC1 protein, P-gp. Transfection of ABCC4 cDNA constructs resulted in low-level resistance to the antifungal agent aureobasidin, also a substrate of ABCC1 (26). ABCC4 expression also correlated negatively with clinical outcome (10). We demonstrated that ABCC4 siRNA-treated cell lines showed minor reductions in paclitaxel resistance. This result is consistent with previous data that reported lower rates of transport of paclitaxel by ABCC4 as compared with ABCC1 (25). The reduction in paclitaxel resistance was unlikely to be caused by changes in ABCC1 expression because we selected ABCC4 specific sequences for the siRNA. In addition, inhibition of ABCC1 will lead to more robust reduction of paclitaxel resistance. The previous studies are consistent with this study, which suggests that ABCC4 does not play as significant a role in paclitaxel resistance as ABCC1 at least in vitro.

Recently, two studies of synthetic siRNA-based suppression of ABCC1 gene expression in multidrug resistance cancer cell lines have been reported (27, 28). These studies targeted ABCC1 and showed that ABCC1 siRNA markedly inhibited the expression of ABCC1 mRNA and P-gp. This study confirms those findings with different target sequences and provides new data comparing synthetic and vector-based siRNA strategies as well as additional studies evaluating ABCC4.
In conclusion, our data confirm the effectiveness of siRNA in inhibiting \textit{ABCB1} expression and the subsequent reversal of resistance to paclitaxel. Moreover, \textit{ABCB4} siRNA also demonstrates very modest inhibition of the paclitaxel-resistant phenotype. These experiments lend credence to the hypothesis that siRNA treatment may represent a new approach for the treatment of \textit{ABCB1}-mediated drug resistance.

References

Molecular Cancer Therapeutics

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