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Effective Knock Down of Very High ABCG2 Expression by a Hammerhead Ribozyme

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Abstract. *Background: Ribozymes are an effective tool to reduce the mRNA levels of specific target genes. Overexpression of the drug transport protein, ABCG2, has been associated with multidrug resistance in cancer cells. Materials and Methods: An expression plasmid encoding a hammerhead ribozyme against the ABCG2 gene was stably transfected into multidrug-resistant MCF7/MX cells that express very high levels of the ABCG2 protein. The effect of the ribozyme was determined by quantitative real-time RT-PCR, Western blot and cytotoxicity assays. Results: The ribozyme reduced ABCG2 mRNA levels to less than 10% of control values, which resulted in the concomitant reduction of ABCG2 protein levels and sensitization of the cells to mitoxantrone and methotrexate. Conclusion: The ribozyme used was highly effective in reducing the expression of its target gene, ABCG2, and was able to modulate the associated multidrug-resistant phenotype.*

Ribozymes are small catalytic RNA molecules that are able to trans-cleave a specific target RNA (1-3). They have been used successfully to knock down the expression of a variety of genes, including drug resistance-related genes, both in cell-free as well as in cellular systems (4-11).

Ribozymes are thought to be highly specific for their target gene by virtue of their hybridization to the target sequence, unlike chemical or pharmacological inhibitors, which often lack specificity and therefore can also affect other unrelated proteins and/or exhibit general toxicity. Thus, ribozymes are valuable tools to study the mechanism of action of proteins. In addition, it has also been suggested that ribozymes could be used pharmacologically to modulate gene expression in tumors. For example, reducing the expression of drug resistance genes, ribozymes might sensitize the tumor cells to chemotherapy and increase the therapeutic efficacy of anticancer drugs.

We have previously reported that a hammerhead ribozyme, RzB1, against the ABCG2 gene displayed *in vitro* cleavage of its target RNA, was able to knock down ABCG2 protein expression and reversed the drug resistance phenotype caused by ABCG2 overexpression (4, 5). However, the cell system used in that study was a drug-resistant stomach cancer cell line, EPG85-257/RNOV, with relatively moderate ABCG2 overexpression. In order to determine whether the RzB1 ribozyme was also effective in other systems with higher ABCG2 expression and thus might be a more generally useful tool to study the function of ABCG2, we expressed the RzB1 ribozyme in a human breast carcinoma cell line, MCF7/MX, which overexpresses ABCG2 6000-fold, or approximately 100-times more than the EPG85-257/RNOV cells (12). The MCF7/MX cells were derived from MCF7 mammary carcinoma cells by step-wise selection in mitoxantrone (13) and are highly resistant to mitoxantrone, topotecan and methotrexate (12-15). Thus, they represent a good model to investigate the efficacy of the anti-ABCG2 ribozyme.

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Materials and Methods

Cells. The human breast carcinoma cell line MCF7/WT, its resistant variant MCF7/MX (13) and its ribozyme-transfected clones were maintained in Improved Minimal Essential Medium, Richter's modification (Irvine Scientific, Irvine, CA, USA), containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 0.01 mg/ml ciprofloxacin (Bayer Pharmaceuticals, West Haven, CT, USA). All tissue cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C.

Drugs. The following stock solutions of drugs were used: 6 mM mitoxantrone in water (Sigma, St. Louis, MO, USA), 10 mM topotecan in water (SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA), and 110.7 mM methotrexate in slightly basic sodium phosphate (Sigma).

Construction of the ribozyme expression vector. The anti-ABCG2 hammerhead ribozyme RzB1 was previously characterized in a cell-

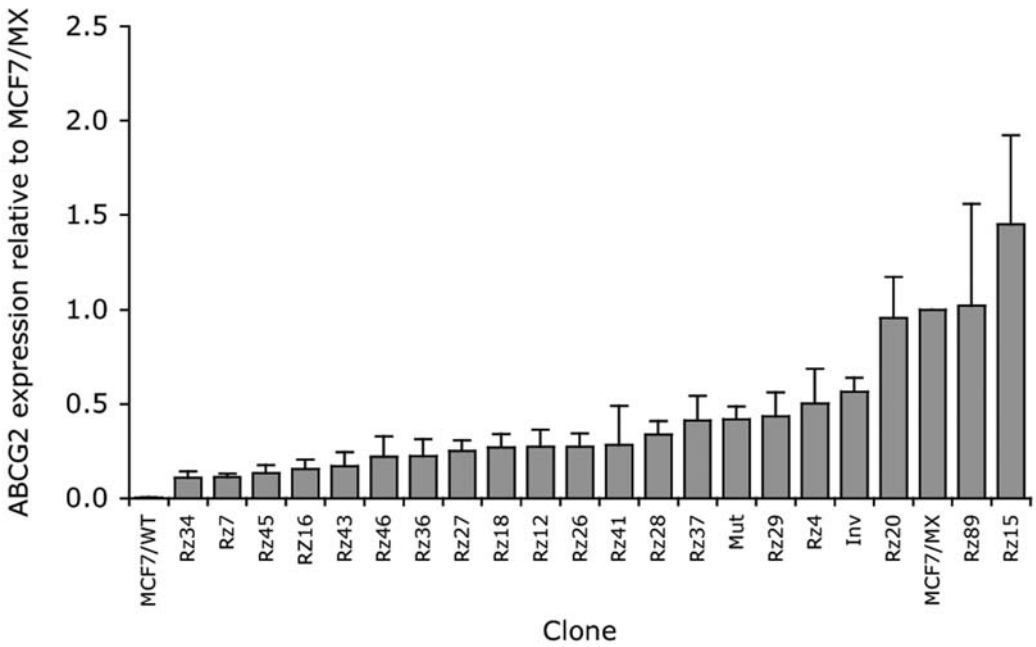

Figure 1. *Relative ABCG2 mRNA expression in RzB1-transfected MCF7/MX cells. ABCG2 mRNA levels were determined by real-time RT-PCR on a LightCycler as described in Materials and Methods. Expression levels were normalized to those in MCF7/MX cells. The values shown are the means±SE from multiple determinations.*

free system and was selected for its high *in vitro* cleavage activity (5). In order to establish an anti-ABCG2-ribozyme expression system, we inserted the ribozyme RzB1-encoding oligodeoxynucleotide 5'-CTGGAACTGATGAGTCCGTGAGGACGAAACATCTGGAG-3' into the multiple cloning site of the eukaryotic expression vector pcDNA3.1/V5-His-TOPO (Invitrogen) (4). As controls, the inverted RzB1 oligodeoxynucleotide (RzB1inv), as well as an oligodeoxynucleotide that encodes a point-mutated, catalytically inactive ribozyme (RzB1mut), were used.

Transfection of the ribozyme and control expression vectors. The expression plasmids pcDNA3.1/RzB1, pcDNA3.1/RzB1inv and pcDNA3.1/RzB1mut were stably transfected into MCF7/MX cells by electroporation at 0.22 kV and 960 μF. After selection in 2 mg/ml G418, visible and clearly separated clones were collected, expanded and maintained in 1 mg/ml G418.

PCR. ABCG2 mRNA expression levels were determined by quantitative real-time RT-PCR on a LightCycler® (Roche, Indianapolis, IN, USA), essentially as previously described (12). Briefly, cDNAs for ABCG2 (using two separate primer sets) and the housekeeping genes porphobilinogen deaminase (PBGD), glyceraldehydephosphate dehydrogenase (GAPDH) and aldolase were amplified using the FastStart DNA Master SYBR Green I reagent kit (Roche). Primers and reaction conditions were as previously reported (4, 12). Expression of the ribozyme was confirmed by amplification of cDNA from transfected cells with primers flanking the ribozyme insertion site in the pcDNA3.1/RzB1 plasmid used for the transfection, essentially as described (4).

Western blotting. ABCG2 protein expression was analyzed by Western blot. Five μg of whole-cell extracts were fractionated by polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Chemicon, Hofheim, Germany) and ABCG2 and glyceraldehyde-3-phosphate dehydrogenase were detected with a polyclonal anti-MXR/ABCG2/ABCP antibody (16) (kindly provided by Dr. Susan Bates, National Cancer Institute, Bethesda, MD, USA) and a monoclonal mouse anti-GAPDH antibody (Chemicon), respectively. Bound antibody was detected by chemiluminescence using the Super Signal Substrate (Pierce, Rockford, IL, USA).

Cytotoxicity assays. The cytotoxicity of mitoxantrone, topotecan and methotrexate in MCF7/WT and MCF7/MX cells, as well as in several separate RzB1, RzB1inv and RzB1mut-transfected clones, was determined using the sulforhodamine B (SRB) method as described previously (4).

Results

Knock down of ABCG2 expression. Following the stable transfection of the RzB1 expression vector into MCF7/MX cells, multiple independent clones were selected, and ABCG2 mRNA levels in each clone were determined by quantitative real-time RT-PCR and compared to those in MCF7/MX cells. As shown in Figure 1, ABCG2 mRNA levels among the clones varied greatly, from unchanged to as low as 10% of the initial level in untransfected MCF7/MX cells. As a result, overexpression of ABCG2 relative to the drug-sensitive MCF7/WT cells was reduced from almost 6000-fold in MCF7/MX cells to approximately 600-fold. Surprisingly, there was also an approximately 50% reduction in ABCG2 transcript levels in clones that had been transfected with either an inverted or a mutant, inactive ribozyme. Though the reasons

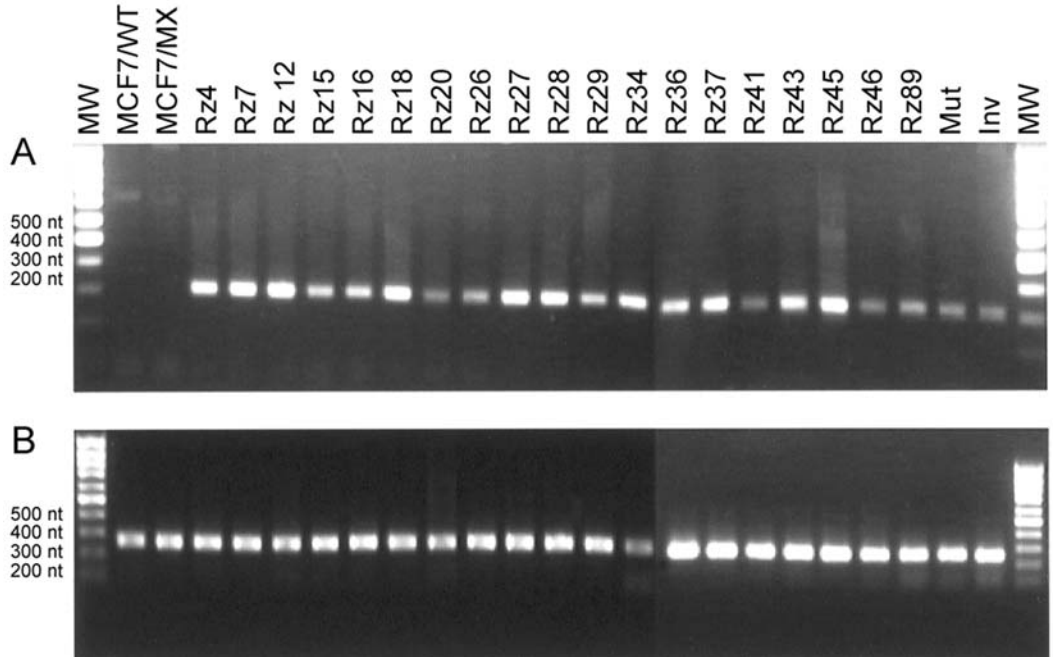

Figure 2. *Ribozyme expression in RzB1-transfected MCF7/MX cells. cDNAs from the RzB1-transfected cells and the non-transfected MCF7/WT and MCF7/MX controls were amplified by regular PCR with A) primers flanking the ribozyme insertion site, and B) primers for the housekeeping gene aldolase.*

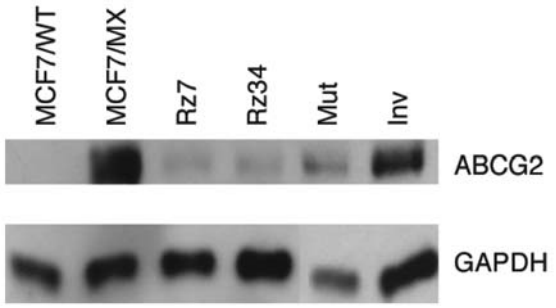

Figure 3. *Western blot for ABCG2. Total cell lysates were analyzed by Western blotting with a polyclonal anti-ABCG2 antibody and a monoclonal GAPDH antibody.*

for this effect are not clear, it is possible that it is caused by binding of the ribozyme arms to their target sequence, followed by degradation of the RNA-RNA hybrid, similar to an antisene effect. In order to confirm that the effects seen were indeed due to the expression of the ribozyme, we also performed RT-PCR for the ribozyme transcript itself by using primers that flank the inserted ribozyme sequence in the pcDNA3.1 plasmid. As shown in Figure 2A, a PCR product was detectable in all transfected clones analyzed, while it was absent in the non-transfected MCF7/WT and MCF7/MX cells. In contrast, a PCR product for the housekeeping gene aldolase was found in all cells, including the non-transfected MCF7/WT and MCF7/MX cells (Figure 2B), thus confirming that there was amplifiable cDNA.

In order to determine whether the reduction in ABCG2 mRNA levels resulted in a concomitant reduction in the amounts of protein, we also determined ABCG2 protein levels by Western blot. As shown in Figure 3, in the two clones with the largest ribozyme effect, Rz34 and Rz7, protein levels were substantially lower than in the MCF7/MX cells. As suggested by the RNA data, there was also some loss of ABCG2 protein in the clones transfected with the inverted and mutated ribozymes, respectively. Note, however, that the control GAPDH band in the Rzmut lane is somewhat weaker than in the other lanes, suggesting that this lane was underloaded, which may have contributed to the apparent reduction of ABCG2 protein. Nevertheless, overall there was a good correlation between mRNA and protein levels, indicating that the ribozyme-mediated reduction in ABCG2 mRNA resulted in a concomitant reduction in protein levels.

Effects of ribozyme expression on drug resistance. To determine whether the ribozyme-mediated knock down of ABCG2 also affected the phenotype of the cells, *i.e.* resulted in a reduction of drug resistance, cytotoxicity assays with mitoxantrone, topotecan and methotrexate were performed with two clones, as well as the control transfectants Rzmut and Rzinv, and the non-transfected MCF7/WT and MCF7/MX cells (Table I). Both clones tested exhibited significantly lower IC_{50}s for mitoxantrone and methotrexate, respectively, corresponding to a 35-70% reduction in relative resistance. In contrast, and somewhat surprisingly, none of the clones tested showed a significant difference in the IC_{50} for topotecan compared to the

Table I. *Cross-resistances of anti-BCRP ribozyme-expressing cells.*

	Mitoxantrone				Topotecan				Methotrexate			
Clone	IC_{50} ±SE* [nM]	*p*-value**	RS	DMF	IC_{50} ±SE* [nM]	*p*-value**	RS	DMF	IC_{50} ±SE* [μM]	*p*-value**	RS	DMF
MCF7/WT	0.22±0.11		0.00026		1.86±0.19		0.0004		0.06±0.01		0.002	
MCF7/MX	856±102	<0.0001[a)]	1.00	1.00	4961±592	<0.0001[a)]	1	1.00	29.5±6.3	0.002[a)]	1.00	1.00
RzB1inv 5	928±128	0.4[b)]	1.08	0.92	3937±471	0.1[b)]	0.79	1.26	19.11±5.50	0.13[b)]	0.65	1.54
RzB1mut 12	813±121	0.4[b)]	0.95	1.05	5767±956	0.2[b)]	1.16	0.86	11.20±2.70	0.02[b)]	0.38	2.63
RzB1clone 7	453±57	0.04[b)]	0.53	1.89	4953±570	0.5[b)]	1.00	1.00	9.32 (8.67, 9.98)		0.32	3.17
RzB1clone 34	395±97	0.009[b)]	0.46	2.17	4445±520	0.3[b)]	0.90	1.12	10.20 (10.75, 9.66)		0.35	2.89

*SE: standard errors are given if at least three independent experiments were performed, otherwise high and low values are shown.
**p-values were determined using the unpaired, one-tailed t-test provided by the Prism3™ software when at least three separate IC_{50} values were available.
[a)]Compared to MCF7/WT. [b)]Compared to MCF7/MX.
RS: Relative sensitivity compared to MCF7/MX cells. DMF: Dose-modifying factor compared to MCF7/MX cells.

non-transfected MCF7/MX cells. Interestingly, some sensitization to methotrexate was also observed in the clones transfected with the mutant and inverted ribozymes, whereas mitoxantrone resistance was not affected in these clones. Thus, anti-ABCG2 ribozyme-mediated knock down of ABCG2 expression resulted in up to 70% loss of mitoxantrone and methotrexate resistance, but did not affect topotecan resistance.

Discussion

Strategies based on the sequence-specific hybridization of short antisense oligo(de)oxynucleotides to a specific target mRNA are potentially very effective in reducing the levels of the corresponding gene product. Various forms of this strategy have been developed, including oligodeoxynucleotides, hammerhead and hairpin ribozymes and, more recently, short interfering RNAs (siRNA). One attractive group of genes to be targeted for knock down have been those associated with multidrug resistance. For example, ribozymes were shown to decrease MDR1-mediated resistance against vincristine and doxorubicin (10, 11), to knock down the lung resistance-related protein (LRP) (6) and to reduce MRP levels (9). Furthermore, recently siRNA was used to knock down MDR1 mRNA levels and to modulate the MDR phenotype (17, 18). Thus, a ribozyme- or siRNA-based approach appears to be an effective and specific way to reverse MDR and to elucidate the role of the genes involved in resistance.

We have previously described a hammerhead ribozyme against the ATP-binding cassette transporter ABCG2 (5) that was effective in reducing ABCG2 expression levels and to resensitizing the cells against mitoxantrone (4). However, the relative overexpression of ABCG2 in the EPG85-257/RNOV cells was moderate (12), and it was not clear whether the RzB1 ribozyme would also be effective against higher levels of ABCG2. In the present study, therefore, in order to further investigate the efficacy of this hammerhead ribozyme, we chose one of several multidrug-resistant cell lines with very high levels of ABCG2 expression (12). Stable transfection of MCF7/MX cells with the anti-ABCG2 ribozyme RzB1 resulted in up to 90% loss of ABCG2 mRNA, which was also reflected in concomitantly lower protein levels. However, the effect of the ribozyme was highly variable between individual clones, despite evidence for the ribozyme transcript in all of them. The exact reasons for this variability are unclear at the moment and need further investigation.

Concomitant with the decrease in ABCG2 mRNA and protein levels, resistance against mitoxantrone and methotrexate was reduced by as much as 70%. Thus, the RzB1 ribozyme appeared highly effective even against very high levels of target mRNA and can be used successfully to reverse ABCG2-mediated drug resistance. However, despite its considerable efficacy, it was insufficient to completely eliminate ABCG2 mRNA or resistance in the MCF7/MX cells. A similar lack of complete resensitization was also observed after siRNA treatment of EPG85-257/RDB cells, which express very high levels of MDR1 (17). Together, these results suggest that, when the target gene expression is very high, additional strategies may need to be employed to achieve a complete depletion of the target gene product. Such strategies might include combining two ribozymes or siRNAs with different target regions on the same gene, or combining a siRNA with a ribozyme.

Various studies have reported that ABCG2 overexpression caused topotecan resistance (19-21). Therefore, it was surprising that the RzB1-expressing clones remained topotecan-resistant despite a clear reduction in ABCG2 levels and in mitoxantrone and methotrexate resistance. In contrast,

cross-resistance against topotecan was completely reversed in EPG85-257/RNOV cells transfected with the same ribozyme RzB1 (Kowalski, unpublished data). However, since there was a difference in the amount of residual ABCG2 between the two cells, it is possible that ABCG2 down-regulation below a certain threshold is required to completely reverse ABCG2-mediated MDR and that this threshold varies for different drugs. At this point it is unclear why different drugs respond differently to varying ABCG2 levels. However, the lack of an effect on topotecan resistance is reminiscent of the situation with flavopiridol. Whereas drug-selected cells that overexpress ABCG2 are clearly flavopiridol-resistant, ectopic expression of ABCG2 by transfection did not result in resistance against this drug (22).

In conclusion, the ribozyme RzB1 has been found to be a very effective tool to reduce ABCG2 expression in different cell lines from tissues of different origin and with widely differing levels of the target gene. This suggests that ribozyme technology may be a promising strategy for prevention and reversion of MDR in oncology, and may be of interest for future gene therapeutic approaches aimed at sensitizing tumors against chemotherapy.

Acknowledgements

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