ORIGINAL

Reduction of expression of the multidrug resistance protein (MRP)1 in glioma cells by antisense phosphorothioate oligonucleotides

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Abstract : The tumor cells' acquisition of resistance to multiple drugs due to overexpression of the multidrug resistance protein (MRP) 1 gene is one of major obstacles in cancer chemotherapy. We have attempted to reverse the multidrug resistance (MDR) phenotype by treating etoposide resistant glioma cell lines (T98G-VP and Gli36-VP) with MRP1 antisense oligonucleotides. 20-mer phosphorothioate oligodeoxynucleotide (0.3μM), complementary to the coding region in the MRP cDNA sequence, could significantly inhibit the growth of multidrug resistant cell lines, T98G-VP and Gli36-VP, cultured in etoposide containing medium. No such effect was observed for the parental T98G and Gli36 cell lines. Further investigations by the reverse transcription-polymerase chain reaction and immunoblotting revealed that antisense oligomer could result in a reduction in the level of MRP1 mRNA, probably through hindering MRP1 gene transcription. This study demonstrates that the antisense oligonucleotides can increase the sensitivity of the tumor cells to the anticancer drug by decreasing the expression of the MRP gene. This strategy may be applicable to cure cancer patients with MRP mediated MDR phenotype.

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INTRODUCTION

The Mr 190,000 multidrug resistance protein (MRP)1 is an identified member of the ATP-binding cassette (ABC) transmembrane transport proteins (1). In addition to the glioma cell lines (T98G and Gli36) from which these proteins were isolated, overexpression of MRP1 has been detected in many multidrug resistant tumor cell lines derived from various tissues and selected in different natural product type chemotherapeutic agents (2).

The efficacy of topoisomerase II inhibitors (e.g., epipodophyllotoxins, aminoacridine, and mitoxantrone) is limited by the occurrence of drug resistance in the tumor cell population. Cellular insensitivity to drugs that stabilize the cleavable complex is frequently expressed as multidrug resistance (MDR)(2). In some cell lines, overexpression of MDR-1/P-glycoprotein or the multidrug resistance associated protein (MRP) 1-5, has been demonstrated and implicated as the mechanism of resistance(2, 3). These proteins are known to confer multidrug resistant phenotypes to the cancer cells. Although the primary structures of P-glycoprotein and MRP share only 15% amino acid homology (1), both P-glycoprotein and MRP are involved in the drug resistance to a similar profile of chemotherapeutic agents that include anthracyclines, vinka alkaloid, and epipodophyllotoxins (3). Another transporter, termed breast cancer-resistance protein (BCRP), also known as Mitoxantrone-resistance protein and placenta ABC protein, was described in breast and colon carcinoma

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cell lines selected for higher-level resistance to the antineoplastic drug, Mitoxantrone (3).

The cells that overexpress P-glycoprotein may be sensitized to the cytotoxic effects of chemotherapeutic agents by the co-administration of a wide variety of the so-called "reversing agents"(4). Two of the most extensively studied reversing agents both *in vitro* and *in vivo* are verapamil, a calcium channel blocker, and cyclosporin A, an immunosuppressive cyclic peptide. However, both verapamil and cyclosporin A are relatively ineffective and/or non-specific chemosensitizers in MRP expressing cell lines, promoting a search for alternative ways to reverse the MRP1-mediated resistance (4). One particularly attractive approach is to use antisense oligonucleotides, because of the potentially high degree of specificity of these reagents. Rather than inhibiting the protein function, the antisense oligonucleotides decrease mRNA and protein synthesis by binding to its complementary nucleic acid target in a sequence specific manner (5-7).

This study demonstrates that the antisense oligonucleotides can increase the sensitivity of the tumor cells to the anticancer drugs by decreasing the expression of the MRP1 gene. This strategy may be applicable to cure the cancer patients with MRP1 mediated multidrug resistance phenotype.

MATERIALS AND METHODS

Oligonucleotides

The oligonucleotides used in this study was described previously (7). The oligonucleotides was 20 bases in length and was phosphorothioate oligodeoxynucleotides. The sequences of antisense oligonucleotides for the MRP1 was as follows : ^{2107}5'-TGCTGTTCGTGCC CCCGCCG-3'

Cell culture and treatment

The T98G-VP and Gli36-VP are multidrug resistant human glioma cell lines overexpressing the MRP, which were derived from the parental T98G and Gli36 cells by culture in etoposide (VP-16)(8).

These cells were grown in 75cm^2 flasks until 70-80% confluent and then were washed twice with serum-free MEM medium before the addition of lipofectin/oligonucleotide complexes. Lipofectin (GIBCO/BRL life technologies, Burlington, Ontario, Canada)(10μg/ml) and the oligonucleotides were allowed to form complexes in serum-free MEM medium at room temperature for 15 min after gentle mixing. The cells were incubated with lipofectin/oligonucleotide at 37°C for 4hr, washed once with MEM/10% fetal bovine serum, and then incubated in fresh MEM/10% fetal bovine serum until harvested.

Reverse transcription and polymerase chain reaction (PCR) analysis for the expression of the MRP 1

Total RNA was isolated from cells by Isogen^R (Nippon gene) according to the manufacture's instructions. RT-PCR was performed using First-Strand cDNA Synthesis Kit^R (Amersham pharmacia biotech). 1μg of total RNA was added to 14μl of RT-mixture. After mixing, the samples were incubated at 37°C for 45 min, 95°C for 5 min and 4°C for at least 5min. 35μl of PCR-mixture containing 10nM primers and Taq DNA polymerase (Amersham pharmacia biotech) was added to RT-products. Initial denaturation for 2 min at 94°C was followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C and a final extension for 10 min at 72°C. The PCR-products were separated on 2% agarose gels, and stained with ethidium (9).

The primer sequences for MRP1 were as follows
5' primer :^{733}5'-CGGAAACCATCCACGACCCTAATCC-3'
3' primer :^{1027}5'-ACCTCCTCATTCGCATCCACCTTGG-3'

Immunoblotting

Immunoblotting for the the MRP1 was performed using detergent-solubilized membrane proteins resolved on a 6% SDS-polyacrylamide. Immunoblotting was performed with a1 : 2000 dilution of a monoclonal MRP1 antibody, kindly provided by Dr. Rik Scheper (Free University Hospital, Netherlands). After three washes in TBS-T, the membranes were incubated with HRP-conjugated anti-mouse secondary antibody at a dilution of 1:1000 for 1 hr. Five washes were then performed and immunolabeled protein was detected by chemiluminescence(2).

Cellular Accumulation for[^{3}H]-VP-16

3.5×10^5 cells were plated in each well of a six-well plate and incubated for 24 hr at 37°C. Growth medium was then replaced with serum-free MEM, and the cells were incubated with 2.6μM [^{3}H]-VP 16 for various times up to 90 min. at 37°C. At the completion of the incubation period, cells were quickly washed three times with ice-cold PBS, lysed with 2ml of 0.05% SDS, and mixed with 3ml of Scintisol (2, 10). Data derived from the drug accumulation analysis was compared using ANOVA. A probability value of less than 0.05 was accepted as statistically significant (2).

Cloning of topoisomerase IIα, MRP1-5, and MDR-1 cDNA by Reverse Transcription-PCR(2)

Synthetic oligonucleotides corresponding to the published cDNA sequence of human MRP1-5were used to isolate by RT-PCR specific products for direct cloning into pGEM-3 z vectors (Promega, Madison, WI). The identity of the cDNA clones was confirmed by direct sequence analysis prior to their use as probes for northern analysis. The sequences of the oligonucleotides used in the RT-PCR were as follows: topoisomerase IIα : -91 5'TGTGGAGAAGCGGCTTGGTC 3' and 449 5'TAGTTACTAGAAGTTAGGAGCT 3' ; MRP 1 : 733 5' CGGAAACCATCCACGACCCTAATCC 3' and 1027 5'ACCTCCTCATTCGCATCCACCTTGG 3'; MRP 2 : 4136 5'CTGCCTCTTCAGAATCTTAG' and 4376 5'CCCAAGTTGCAGGCTGGCC' ; MRP 3 : 5 'GATACGCTCGCCACAGTCC3' and 5'CAGTTGG CCGTGATGTggCTG 3' ; MRP 4 : 5'CCATTGAAG ATCTTCCTGG3' and 5'GGTGTTCAATCTGTGTGC 3' ; MRP 5 : 5'GGATAACTTCTCAGTGGG 3'and 5 'GGAATGGCAATGCTCTAAAG 3' ; MDR-1 : 352 5'CAGTGTTTGCCATAGTATTTTCAAGGATTG 3' and 741 5'CCCTTTAACACTAGAAGCATCAC 3'.

Northern Blotting

Total RNA(20μg/lane) was separated on a 6% formaldehyde gel and transferred to Hybond N+ with 10 X SSC. The membranes were hybridized at 42°C overnight with the radiolabeled probe in Hybrisol I (Oncor, Gaithersburg, MD)[2]. We used the RNA of ZR-VP 13that overexpressed all drug resistant genes (MDR-1, MRP 1-5, topoisomerase II α) as positive control (2).

Cytotoxicity Assays

Cytotoxicity assays were performed as previously described (11). Briefly, cells were plated at 10000 per well in 96-well microtiter plates with various concentrations of the anticancer drugs [VP-16, adriamycin, vincristine, cisplatin, and1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU)] and were cultured for 2 days. Then the cells were incubated with 0.2 mg/ml of MTT in MEM for 4 hr, and the formazan product was dissolved in dimethyl sulfoxide and its absorbance at 540 nm was measured. The cytotoxic effects of the drugs were expressed quantitatively as the dose required to kill 50% of the cells (IC_{50}).

RESULTS

Table1 depicts the cross-resistance profile of the two sublines to various chemotherapeutic agents. Cross-resistance to two topoisomerase II poisons (VP-16, adriamycin) was observed in these drug resistant cells with little cross resistant to non-topoisomerase II poisons, Vincristine, cisplatin, and ACNU. Resistant sublines treated with the MRP1 antisense oligonucleotide displayed an enhanced sensitivity to topoisomerase II inhibitors, VP-16 and Adriamycin.

When expressions of topoisomerase IIα, MRP1-5, and MDR-1 were examined, the results shown in Fig.1 and Table2 were obtained. Northern analysis showed that these resistant cells the overexpress MRP1, but do not overexpress MDR-1 or MRP1-5 except 1. Moreover, in the results of northern blot analysis of mRNA for topoisomerase IIα and topoisomerase II activity in decatanation assay using kinetoplast DNA (data not shown), identical results are observed in parental cell lines and their resistant cell lines, suggesting that alterations in topoisomerase II do not account for the resistance in these cells.

The VP-16 accumulation study was carried out in the parental cell lines and their drug resistant sublines (Fig.2). Figure 2 demonstrates the ^{3} H-VP-16 accumulation in the parental cells and the drug resistant sublines. Accumulation over 45 min. period is displayed as p mol of VP-16/10^{7}cells. ^{3}H-VP-16 accumulations for the resistant sublines relative to parental cells were T 98 G-VP, 48% and gli 36-VP, 58%. Increased accumulation was observed in the two resistant sublines treated with the MRP1 antisense oligonucleotide, with partial restoration of levels to that of the parental cells (T98 G-VP with MRP1 antisense treatment, 96% of parental T 98 G ; and Gli36-VP with the MRP1 antisense treatment, 94% of parental Gli 36).

Figure 3 depicts concentration-dependent reduction of MRP1 mRNA and protein in VP-16 resistant cell lines by a treatment with the MRP1 antisense oligonucleotide. The T98G-VP and Gli-VP cells were exposed for 4 hr to selected concentrations of the MRP1 antisense oligonucleotide. The MRP 1 mRNA was isolated 24 hr after treatment, and RT-PCR and immunoblotting were performed. A concentration-dependent decrease in the MRP1 mRNA was observed after a single treatment of the T98G-VP with the MRP antisense oligonucleotide. A significant decrease of the MRP mRNA and protein were observed at 0.1μM, and virtually no MRP mRNA was detectable at oligonucleotide concentration of 0.3 μM (upper panel). Similar results were found in the experiment using the Gli36-VP cell line. Significant reduced MRP mRNA and protein were detectable at oligonucleotide concentration of 0.3μM (lower panel).

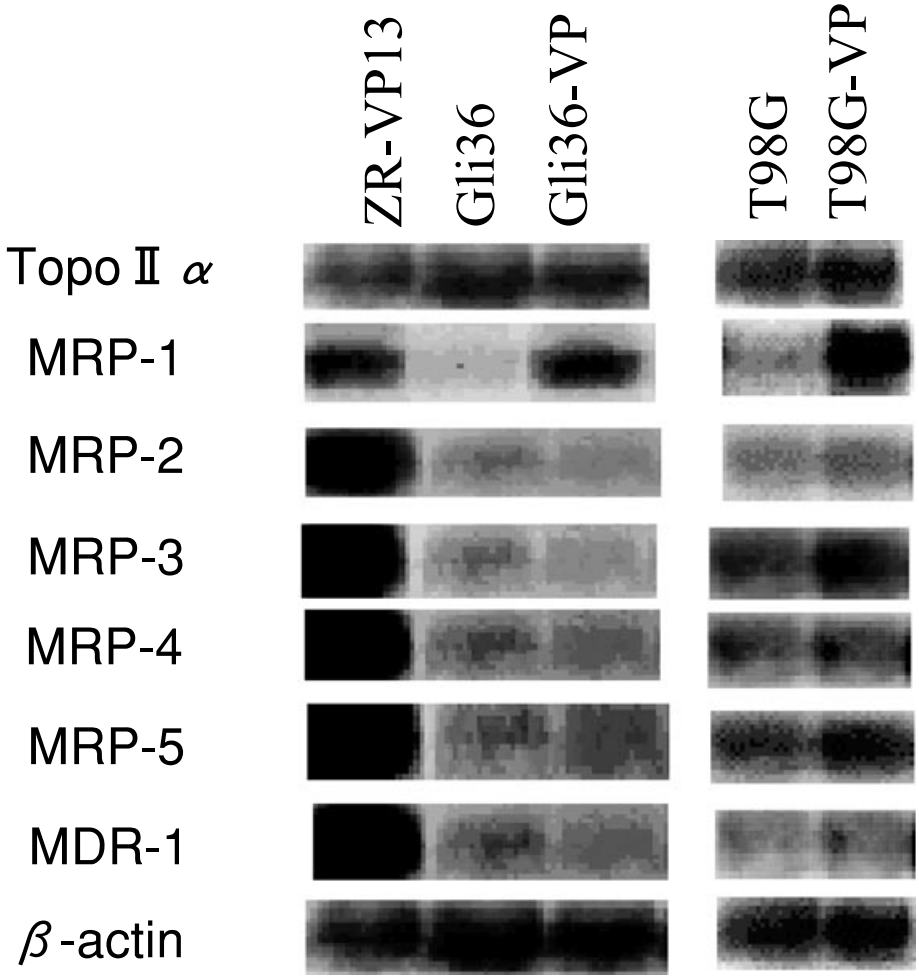

Figure 1：Northern blot analysis of topoisomerase IIα(4.5 KB), MRP 1-5(4.5 KB, 4.4 KB, 4.7 KB, 4.3 KB, 6.5 KB), and MDR-1 (4.5 KB) expression in drug resistant sublines and their parental cell lines. The RNA of ZR-VP13 that overexpressed all drug resistant genes (MDR-1, MRP 1-5, topoisomerase Ⅱ α) was used as positive control. β-actin was used as internal control.

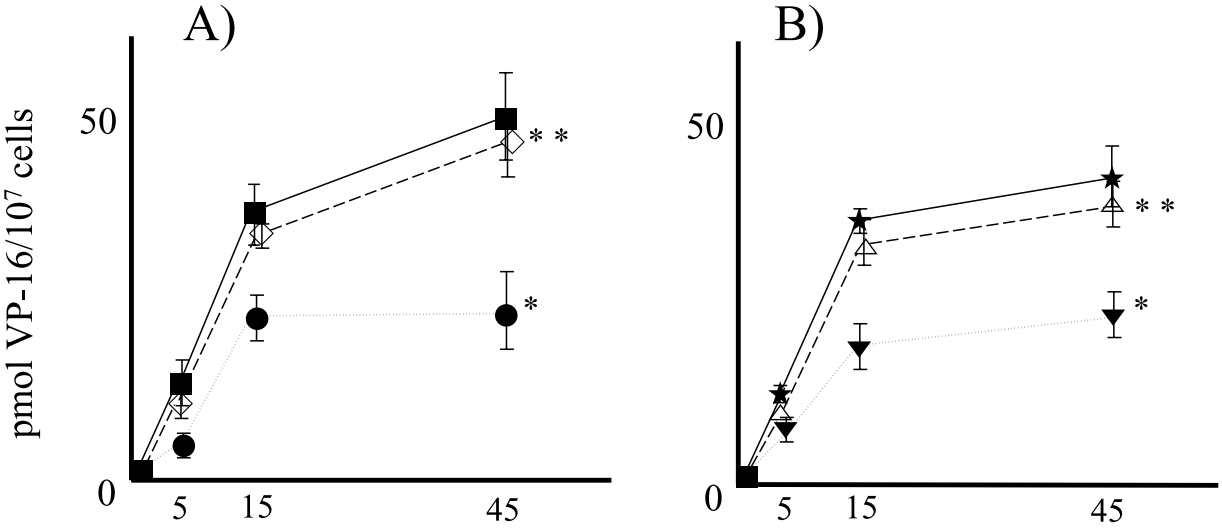

Figure 2 : VP-16 accumulation study was carried out in parental cell lines and their drug resistant sublines (A; T98G, B; Gli36).
* significantly different ($p<0.05$) between resistant cell lines and their parental cell lines(incubation time at 45 min).
* * significantly different ($p<0.05$) between resistant cell lines and their own transfected MRP 2 antisense cDNA (incubation time at 45 min)
left panel : ■ ; T98G, ● ; T98G-VP, ◇ ; T98G-VP with MRP 1 antisense treatment
right panel : ★ ; Gli 36,▼ ; Gli 36-VP, △ ; Gli 36-VP with MRP 1 antisense treatment

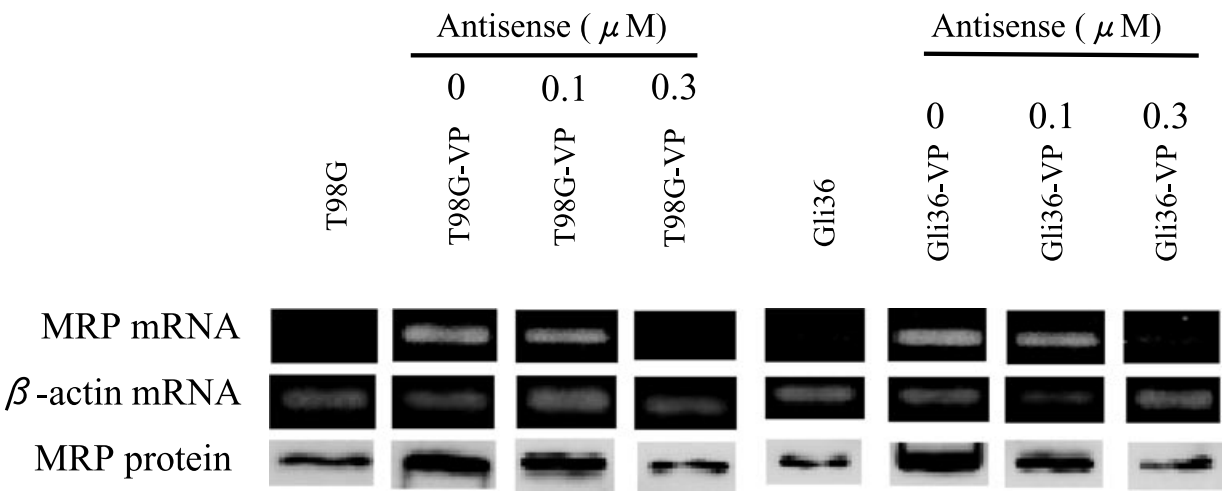

Figure 3 : Concentration-dependent reduction of MRP1 mRNA and protein in etoposide resistant cell lines by a treatment with the MRP1 antisense oligonucleotide. Upper panel; a concentration-dependent decrease in the MRP1 mRNA and protein were observed after a single treatment of T98G-VP and Gli 36-VP with MRP antisense oligonucleotides.

Table 1. Cross resistace profile of the drug resistant cell lines

1) Gli36-VP

	IC_{50} Gli 36	IC_{50} Gli-VP(-)[b]	ratio[a] Gli-VP(-) / Gli 36	IC_{50} Gli-VP(+)[c]	ratio[a] Gli-VP(+) / Gli 36
VP 16[d]	1.5±0.4	37.5±3.5	25	2.5±1.2	1.7
ADM[e]	0.031±0.01	0.37±0.08	12	0.043±0.22	1.4
VCR[f]	0.31±0.13	0.39±0.21	1.3	0.29±0.16	0.9
CDDP[g]	220±55	290±78	1.3	310±98	1.4
ACNU[h]	420±120	480±140	1.1	450±100	1.1

IC_{50} : ng/ml

2) T98G-VP

	IC_{50} T98G	IC_{50} T98G-VP(-)[b]	ratio[a] T98G-VP(-) / T98G	IC_{50} T98G-VP(+)[c]	ratio[a] T98G-VP(+) / T98G
VP 16[d]	7.5±2.4	137.5±33.5	18	8.5±5.2	1.1
ADM[e]	0.081±0.01	0.47±0.08	6	0.096±0.02	1.2
VCR[f]	1.31±0.33	1.39±0.51	1.1	1.39±0.36	1.1
CDDP[g]	420±155	490±178	1.2	510±198	1.2
ACNU[h]	390±220	480±140	1.2	450±178	1.2

IC_{50} : ng/ml

a : ratio of the individual cell lines
b : without MRP antisense oligonucleotides
c : with MRP antisense oligonucleotides
d : etoposide, e : adriamycin, f : vincristine, g : cispatin, h : 1-(4-aminio-2-methyl-5-pyrimidinyl) methyl-3-(2-choloroethyl)-3-nitrosourea

Table 2. Northern blot analysis of topoisomerase IIα, MRP1-5, and MDR-1 expression in drug resistant sublines and their parental cell lines

	ZR-VP13	T98G	T98G-VP	Gli36	Gli36-VP
Topo II α	100	114	108	107	112
MRP-1	100	3	105	8	117
MRPP-2	100	8	4	9	10
MRP-3	100	11	9	16	18
MRP-4	100	12	13	18	19
MRP-5	100	12	14	19	20
MDR-1	100	11	9	16	18

Densitometric quantitations of drug resistance gene expressions were performed by comparing with those in ZR-VP13 cells

DISCUSSION

Since the development of MDR by cancer cells still represents one of the major reasons for anticancer chemotherapy failure (12-18), the aim of the present study was to attempt modulation and eventually reversal of MDR in the glioma cell lines *in vitro*, using antisense oligonucleotides to targeted to the MRP1 mRNA. In a previous study, the 16 oligonucleotides were screened initially for their ability to decrease the MRP1 protein and mRNA levels in MRP1-overexpressing H69AR cells (7). One of the oligonucleotides, which we used in this study, was complementary to nucleotide 2107-2126 of MRP1 mRNA and reproducibly the most effective in lowering the MRP1 protein and mRNA levels. It was also demonstrated that the decrease in MRP1 mRNA results from site specific cleavage, consistent with an RNAseH-dependent antisense mechanism (10, 19-21).

The ability of MRP1 antisense oligonucleotide to increase VP-16 cytotoxicity in the T98G-VP and Gli36-VP cells and thus to reverse the MDR was assessed in combination experiments in which the T98G-VP and Gli36-VP cells were first exposed to MRP antisense oligonucleotide and then to VP-16. Treatment with VP-16 alone had modest effects on the T98G-VP and Gli 36-VP cell proliferation. The *in vitro* restoration of VP-16 sensitivity in T98 G-VP and Gli36-VP was confirmed by the VP-16 IC_{50} value for each treatment. A strong decrease of the IC_{50} value was observed in the T98G-VP and Gli36-VP cells treated with combination of MRP antisense oligonucleotide plus VP-16. Noteworthy, the IC_{50} value of the resistant cells exposed to antisense oligonucleotide plus VP-16 was essentially identical to that of the T98G and Gli 36 parental cells exposed to the drug alone, consistent with a complete restoration of the VP-16 accumulation in T98G-VP and Gli36-VP cells.

Transfection experiments with MRP expression vectors have shown that MRP can confer resistance to adriamycin and VP-16(22). It has been shown that MRP gene encodes glutathione S-conjugate export carrier, and that the molecules which are transported by MRP include adriamycin glutathione conjugates and glucuronosyl VP-16. These reports are consistent with our present study using the MRP antisense oligonucleotide.

MRP expression has also been observed in other clinical cancers, including colorectal carcinoma, lung cancer, lung cancer, breast carcinoma, retinoblastoma, and acute leukemia. Regarding the nervous system, it has been reported that MRP mRNA and protein are not detectable in the brain, and glioma cell lines with higher MRP mRNA levels show increased drug resistance. Since all the present clinical specimens examined were primary tumors, we considered that the MRP expression was concerned with intrinsic drug resistance as well as acquired drug resistance (19, 22). In an attempt to correlate the anti-proliferative effect of the MRP1 antisense oligonucleotide with the levels of MRP1 mRNA, we evaluated the MRP1 mRNA and protein expression by RT-PCR and immunoblotting in the T98G-VP and Gli36-VP cells immediately after treatment with MRP1 antisense oligonucleotide plus VP-16. In comparison to the untreated cells, the cells treated with the MRP1 antisense oligonucleotide exhibited a marked reduction of the MRP1mRNA and protein. The level of β-actin mRNA were essentially unchanged in the cells receiving different treatments, confirming the specificity of the effects induced by the MRP1 antisense oligonucleotide.

In conclusion, our studies raise the possibility that antisense oligonucleotide targeted to MRP1 mRNA might be useful in the attempt to reverse the MDR in the cancer patients.

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