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Reduction of Expression of the Multidrug Resistance Protein (MRP) in Human Tumor Cells by Antisense Phosphorothioate Oligonucleotides

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ABSTRACT. Multidrug resistance protein (MRP) is a member of the ATP-binding cassette superfamily of transport proteins which has been demonstrated to cause multidrug resistance when transfected into previously sensitive cells. Sixteen eicosomeric oligonucleotides complementary to different regions along the entire length of the MRP mRNA reduced MRP mRNA and protein levels in drug-resistant small cell lung cancer cells that highly overexpress this protein. In MRP-transfected HeLa cells that express intermediate levels of MRP, one oligonucleotide, ISIS 7597, targeted to the coding region of the MRP mRNA, decreased the levels of MRP mRNA to <10% of control levels in a concentration-dependent manner. This effect was rapid but transient with a return to control levels of MRP mRNA 72 hr after treatment. A double treatment with ISIS 7597 produced a sustained inhibition, resulting in a greater than 90% reduction in MRP mRNA for 72 hr and a comparable decrease in protein levels. Increased sensitivity to doxorubicin was observed under these conditions. Northern blotting analyses using two DNA probes corresponding to sequences 5′ and 3′ of the ISIS 7597 target sequence, respectively, revealed the presence of low levels of two smaller sized RNA fragments as expected from an RNase H-mediated mechanism of action of the antisense oligonucleotide. These studies indicate that a specific reduction in MRP expression can be achieved with antisense oligonucleotides, a finding that has potential implications for the treatment of drug-resistant tumors. BIOCHEM PHARMACOL 51;4:461–469, 1996.

KEY WORDS. antisense; RNase H; multidrug resistance; MRP; chemotherapy

The M_r 190,000 MRP‖ is a recently identified member of the ABC superfamily of transmembrane transport proteins [1, 2]. In addition to the small cell lung cancer cell line (H69AR) from which it was isolated, overexpression of MRP has been detected in many multidrug-resistant tumor cell lines derived from a variety of tissues and selected in different natural product type chemotherapeutic agents [1, 3–10]. Cells transfected with a full-length MRP cDNA show increased resistance to a wide range of drugs, in addition to trivalent and pentavalent arsenical and antimonial oxyanions [2, 11]. Thus, MRP and the well-characterized (but only distantly related) P-glycoprotein encoded by the *MDR1* gene are the only human members of the ABC superfamily which are presently known to confer multidrug resistance [12–15].

Cells that overexpress P-glycoprotein may be sensitized to the cytotoxic effects of chemotherapeutic agents by the co-administration of a wide variety of so-called "reversing agents" [16–18]. Two of the most studied reversing agents both *in vitro* and *in vivo* are verapamil, a calcium channel blocker, and cyclosporin A, an immunosuppressive cyclic peptide [17]. However, both verapamil and cyclosporin A are relatively ineffective and/or non-specific chemosensitizers in MRP expressing cell lines [11, 19–21], prompting a search for alternative ways to reverse MRP-mediated resistance. One particularly attractive approach is to use antisense oligonucleotides, because of the potentially high degree of specificity of these reagents. Rather than inhibiting protein function, an antisense oligonucleotide decreases protein synthesis by binding to its complementary nucleic acid target in a sequence specific manner.

Antisense oligonucleotides have been used previously to inhibit the synthesis of P-glycoprotein. In these studies, various phosphodiester [22–24], methylphosphonate [25], and phosphorothioate [26, 27] oligonucleotides have been shown to decrease *MDR1* mRNA and protein. Increased efficacy of a partially modified phosphodiester *MDR1* specific antisense oligonucleotide was observed when it was delivered with liposomes [28]. Although these studies suggest that oligonucleotides may be effective inhibitors of multidrug resistance, an antisense mechanism of inhibition was not demonstrated clearly.

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‖ *Abbreviations:* MRP, multidrug resistance protein; ABC, ATP-binding cassette; SSC, standard sodium citrate; NGS, normal goat serum; and TBST, Tris-buffered saline, 0.05% Tween 20.

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In the present study, a panel of antisense phosphorothioate oligonucleotides complementary to both coding and non-coding regions of MRP mRNA was screened. One oligonucleotide, ISIS 7597, was chosen for further characterization on the basis of its ability to decrease both MRP protein and mRNA levels. Subsequently, the effects of ISIS 7597 on MRP mRNA and protein levels were studied to determine schedules for maximal suppression. In this way, we were able to inhibit MRP expression by more than 90%. We were also able to demonstrate that the decrease in MRP mRNA results from site specific cleavage, consistent with an RNase H-dependent antisense mechanism.

MATERIALS AND METHODS

Oligonucleotides

The oligonucleotides used in this study were complementary to various regions of the MRP mRNA sequence and are listed in Table 1. Oligonucleotides were 20 bases in length and were synthesized as described previously [29, 30]. Fifteen were phosphorothioate oligodeoxynucleotides, and one (ISIS 7607) was a 2′-*O*-methyl derivative with the same sequence as ISIS 7608. ISIS 9658 was a sense control oligonucleotide with a sequence complementary to ISIS 7597.

Cell Culture and Treatment

H69AR is a multidrug-resistant human small cell lung cancer cell line overexpressing MRP, which was derived from parental H69 cells by culture in doxorubicin [31]. T5 cells are a population of transfected HeLa cells that overexpress MRP and were obtained by stable transfection with an MRP cDNA expression vector, pRc/CMV-MRP1. These cells were maintained in medium containing geneticin as described previously [2, 11].

Cells were grown in 75 cm^2 flasks until 70–80% confluent and then were washed twice with serum-free RPMI 1640 medium before the addition of lipofectin/oligonucleotide complexes. Lipofectin (GIBCO/BRL Life Technologies, Burlington, Ontario, Canada) (5 μg/mL for HeLa T5 cells; 10 μg/mL for H69AR cells) and the oligonucleotide (at concentrations indicated in the figures) were allowed to form complexes in serum-free RPMI 1640 medium at room temperature for 15 min after gentle mixing. The cells were incubated with lipofectin/oligonucleotide at 37° for 4 hr, washed once with RPMI 1640/10% fetal bovine serum, and then incubated in fresh RPMI 1640/10% fetal bovine serum until harvested.

Measurement of MRP mRNA Levels

Total RNA was isolated from cells by lysis in Trizol reagent (GIBCO/BRL Life Technologies) according to the manufacturer's instructions. RNA (5 μg) was analyzed on 1% agarose gels containing formaldehyde and transferred by pressure blotting (Stratagene) to nylon membranes (Zeta-Probe, Bio-Rad, Mississauga, Ontario, Canada). Blots were hybridized with MRP DNA probes, corresponding to nucleotides 1219–1327 (mrp 1108), 1158–3067 (mrp 20) or 4080–5011 (mrp 10.1) (nucleotide numbering relative to the start of coding sequence) [1, 32]. To assess the equivalence of RNA loading, blots were also probed with GAPDH cDNA. The cDNA probes were radiolabeled with [α-^{32}P]dATP by random priming (BRL Random Primers DNA labeling system) (GIBCO/BRL Life Technologies). Blots were hybridized overnight at 42° in a solution of 5× SSC, 5× Denhardt's solution, 1% SDS, 50% formamide and 100 μg/mL herring testis DNA. They were then washed for 30 min at room temperature in 0.5× SSC, followed by two washes of 30 min at 52° in 0.1× SSC/0.1% SDS and exposure to Kodak X-Omat AR film. Relative levels of mRNA were estimated by densitometric analysis of the autoradiographs and expressed as a percentage of the values obtained with the serum-free (SF-CON) control (Molecular Dynamics).

Measurement of MRP Protein Levels in H69AR Cells by Cell ELISA

H69AR cells were treated twice with the various oligonucleotides listed in Table 1 at a concentration of 0.5 μM for 4 hr with an interval of 48 hr between treatments. Cells were harvested 20 hr after the second treatment, washed twice with PBS, and resuspended at 5×10^4 cells/mL; 100 μL of the cell suspension was dispensed into each well of a 96-well microtiter plate. Plates were dried overnight at 37° and used immediately or stored at 4° and used within 1 week. Cells were rehydrated with TBST and blocked for 1 hr at room temperature with 1% BSA and 5% NGS in TBST (blocking solution). MRP-specific antisera (MRP-L) [33] was then added at 1:5 final dilution in blocking buffer for 1.5 hr at room temperature. Binding of the MRP-specific antisera was detected using a goat anti-rabbit alkaline phosphatase conjugate with *p*-nitrophenyl-phosphate as substrate. To control for possible variation in cell numbers, cell ELISAs were also performed with the annexin II-specific monoclonal antibody 3.186 [34, 35], and the ratio of MRP levels to annexin II was determined.

Immunoblot Analysis of MRP Protein Levels

The relative amounts of MRP protein were assessed by immunoblot analysis of total cell extracts and membrane-enriched fractions. Pellets of T5 cells (50×10^6/mL) were resuspended in 10 mM Tris-HCl, pH 7.5, containing 10 mM KCl, 1.5 mM $MgCl_2$ and a mixture of protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 50 μg/mL antipain, 2 μg/mL aprotinin, 200 μg/mL EDTA, 200 μg/mL benzamidine, 0.5 μg/mL leupeptin, and 1 μg/mL pepstatin). After 10 min, the suspension was homogenized in a chilled Tenbroeck homogenizer with 80 strokes of the pestle. The homogenate was centrifuged at 800 *g* at 4° for 15 min to remove nuclei and remaining intact cells. A membrane-enriched fraction was prepared by ultracentrifugation of the supernatant at 100,000 *g* at 4° for 20 min. The pellets were resuspended in 10 mM Tris–HCl, pH 7.5, containing 125 mM sucrose.

For SDS–PAGE and immunoblotting, samples were re-

solved on a 7% separating gel and a 4% stacking gel. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Mississauga, Ontario, Canada). For detection of MRP, blots were incubated with MRP-specific monoclonal antibody QCRL-1 [36]. Antibody binding was visualized with horseradish peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence detection (Amersham, Oakville, Ontario, Canada) and exposure on Kodak X-OMAT film.

Chemosensitivity Testing

Drug sensitivity was measured using a tetrazolium salt-based cytotoxicity assay [19]. Briefly, cells were dispensed in a volume of 500 μL at 12×10^4 cells/well in a 24-well plate and incubated at 37° for 20–22 hr to allow the cells to attach. The medium was removed, the cells were washed twice with serum-free medium, and the lipofectin-oligonucleotide complex was added to the wells. In control wells, only serum-free medium was added. The cells were incubated for 4 hr, washed once with complete medium, and then incubated again for 48 hr. At this time, cells were treated again with oligonucleotide as before. At the end of the second treatment, the cells were washed once with complete medium and allowed to recover for 1 hr; then doxorubicin (Sigma) was added to the wells at twice the final desired concentration in a volume of 500 μL. Following incubation for a further 48 hr at 37°, 500 μL medium was removed from each well, 100 μL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL in PBS) was added, and the plates were incubated for 3 hr at 37°. The reaction was stopped by the addition of 1 N HCl:isopropanol (1:24) (1 mL/well) followed by thorough mixing. Absorbance at 540 nm was determined, and doxorubicin cytotoxicity was expressed as a percentage of the absorbance values obtained with cells receiving no doxorubicin. All drug concentrations were tested in duplicate wells in each experiment, and four independent experiments were performed.

RESULTS

Effects of Oligonucleotides on MRP Protein and mRNA Levels

The 16 oligonucleotides (Table 1) were screened initially for their ability to decrease MRP protein levels in MRP-overexpressing H69AR cells. We have determined previously that the half-life of the mature M_r 190,000 MRP is approximately 19 hr [33]. In view of the stability of the protein, we anticipated that a single treatment with oligonucleotide might not be sufficient to decrease protein levels substantially. Consequently, we initially used a double oligonucleotide treatment over 3 days. These experiments were carried out with 0.5 μM oligonucleotide because higher concentrations (>1 μM) were found to be mildly toxic in combination with lipofectin. After two 4-hr treatments with oligonucleotides, separated by a 48-hr interval, MRP protein levels in H69AR cells were found to vary considerably depending on the oligonucleotide used (Table 1). MRP protein levels were estimated using a whole cell ELISA with affinity-purified MRP polyclonal antiserum 20 hr after the second treatment [33, 37]. A monoclonal antibody against annexin II was used to normalize for any variation in

TABLE 1. Effect of phosphorothioate oligonucleotides on MRP protein and mRNA levels in H69AR cells

Oligonucleotide No.	Sequence (5′-3′)	mRNA target	% Control MRP	
			Protein*	mRNA†
ISIS 7607	CGGGGCCGCAACGCCGCCTG	Cap site‡	91 ± 17	150
ISIS 7608	CGGGGCCGCAACGCCGCCTG	Cap site	95 ± 25	17
ISIS 7606	GGTGATCGGGCCCGGTTGCT	Non-coding	68 ± 6	24
ISIS 7595	CCGGTGGCGCGGGCGGCGGC	5′-Untranslated	69 ± 10	4
ISIS 7592	AGCCCCGGAGCGCCATGCCG	AUG codon	74 ± 7	1
ISIS 7593	TCGGAGCCATCGGCGCTGCA	Coding	80 ± 10	7
ISIS 7594	GGCACCCACACGAGGACCGT	Coding	79 ± 25	3
ISIS 7597	TGCTGTTCGTGCCCCCGCCG	Coding	51 ± 12	10
ISIS 7598	CGCGCTGCTTCTGGCCCCCA	Coding§	56 ± 22	12
ISIS 7599	GCGGCGATGGGCGTGGCCAG	Coding	73 ± 20	13
ISIS 7600	CAGGAGGTCCGATGGGGCGC	Coding	95 ± 15	13
ISIS 7601	GCTCACACCAAGCCGGCGTC	Stop codon	78 ± 12	5
ISIS 7603	AGGCCCTGCAGTTCTGACCA	3′-Untranslated‖	95 ± 13	50
ISIS 7605	CTCCTCCCTGGGCGCTGGCA	3′-Untranslated‖	74 ± 18	9
ISIS 7602	ACCGGATGGCGGTGGCTGCT	3′-Untranslated‖	73 ± 8	13
ISIS 7604	CGCATCTCTGTCTCTCCTGG	3′-Untranslated‖	77 ± 20	30

Oligonucleotides were tested for their ability to inhibit immunoreactive MRP protein synthesis in H69AR cells by whole cell ELISA and to decrease the expression of the MRP mRNA by northern analysis (see Materials and Methods). Oligonucleotide sequences are shown 5′-3′, and the approximate target on the human MRP mRNA is indicated. The values for MRP protein and mRNA levels are expressed as percent of controls (no oligonucleotide treatment). Values of protein levels are means ± SD of 3–4 experiments.

* Cells were treated twice with 0.5 μM oligonucleotide in lipofectin for 4 hr with a 48-hr interval between treatments. Cells were collected 20 hr after the second treatment for the measurement of protein level by cell ELISA.

† mRNA levels were determined 24 hr after a single treatment with 0.3 μM oligonucleotide.

‡ 2′-O-Methyl derviative.

§ NH_2-proximal nucleotide binding domain.

‖ Sequence present in H69AR MRP mRNA but not in vector-encoded MRP mRNA of transfected T5 cells.

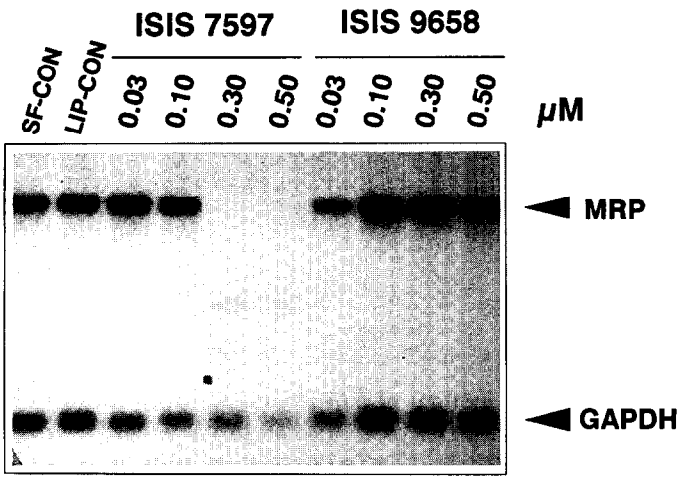

FIG. 1. Concentration-dependent reduction of MRP mRNA levels in transfected HeLa T5 cells by a single treatment with ISIS 7597. MRP-transfected T5 cells were exposed for 4 hr to selected concentrations of MRP antisense oligonucleotide ISIS 7597 and its corresponding sense oligonucleotide, ISIS 9658, complexed with lipofectin. MRP mRNA was isolated 24 hr after treatment, and northern analyses were performed. Relative MRP mRNA levels were estimated by densitometry and normalized to the GAPDH mRNA signal. Results shown are from a single experiment, and similar results were found in a second experiment. LIP-CON = lipofectin control.

cell numbers [34, 35]. Two oligonucleotides were reproducibly the most effective at lowering MRP protein levels. These were ISIS 7597 and 7598, which are complementary to nucleotides 2107–2126 and 2503–2522 of MRP mRNA, respectively.

The sixteen oligonucleotides were also screened for their ability to decrease MRP mRNA levels in H69AR cells (Table 1). After a single treatment, only one oligonucleotide had no effect. This oligonucleotide, ISIS 7607, is a 2′-O-methyl oligoribonucleotide. Because it does not form a hybrid that is a substrate for RNase H, a decrease in mRNA levels was not necessarily expected in the 4-hr time frame of the experiment [38]. As shown in Table 1, the phosphorothioate oligodeoxynucleotides all decreased MRP mRNA levels to various degrees, and ISIS 7597 and 7598 were among the most efficacious. Lipofectin by itself had no effect.

Subsequent experiments focused solely on ISIS 7597 (and its corresponding sense control, ISIS 9658) because of its efficacy in decreasing both MRP mRNA and protein levels. ISIS 7598 was not used further because it is complementary to a region of MRP mRNA encoding the NH_2-proximal nucleotide binding domain. This is the most conserved region of members of the ABC transporter superfamily. Consequently, the oligonucleotide could potentially affect expression of other proteins containing similar nucleotide binding domains and thus be less specific. We have shown previously that resistance in H69AR cells is multifactorial [39–41]. For this reason, subsequent experiments with ISIS 7597 were carried out in transfected HeLa T5 cells where resistance is known to be solely attributable to overexpression of MRP [2, 11, 33].

Effect of Single Treatment with ISIS 7597 on HeLa T5 Cells

A concentration-dependent decrease in MRP mRNA was observed after a single treatment of transfected T5 cells with ISIS 7597 (Fig. 1). A significant decrease was observed at 0. 1μM, and virtually no MRP mRNA was detectable at oligonucleotide concentrations of 0.3 and 0.5 μM. The sense control ISIS 9658 had no effect at concentrations up to 0.5 μM. The >90% decrease in mRNA levels after treatment with 0.5 μM ISIS 7597 was transient (Fig. 2). Maximal levels of mRNA inhibition were reached at 4 hr and maintained for 24 hr after treatment. MRP mRNA levels returned to approximately 70 and 100% of those in untreated controls after 48 and 72 hr, respectively (Fig. 2). In the same experiment, the levels of MRP protein were estimated by immunoblot analysis. As was the case for mRNA levels, ISIS 7597 (0.5 μM) decreased MRP protein levels transiently and maximally to 30% of control levels after 48 hr (Fig. 2). This reduction in protein levels is consistent with its previously determined half-life and the kinetics with which MRP mRNA levels are depleted and recover [33].

Effect of a Double Treatment with ISIS 7597 on HeLa T5 Cells

The relatively long half-life of MRP suggested that it would be necessary to suppress mRNA levels for at least 2.5 to 3 days to be sure of decreasing protein levels approximately 10-fold. In an attempt to achieve this level of response, cells were retreated 48 hr after initial exposure to oligonucleotide. When levels of MRP mRNA were determined 24 hr after a second

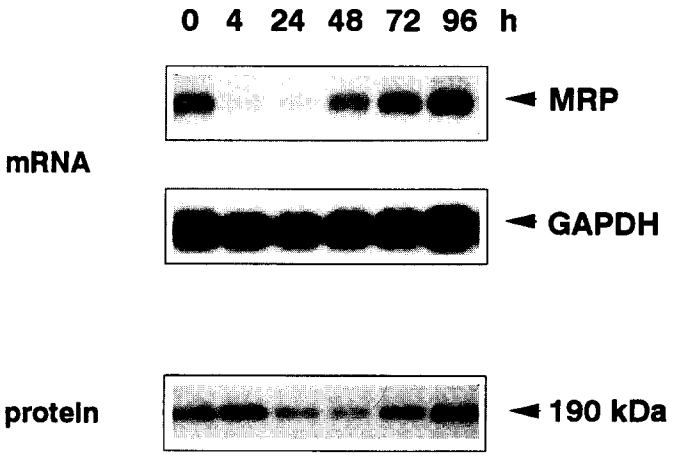

FIG. 2. Time-dependent reduction of MRP mRNA and protein levels in transfected HeLa T5 cells by a single treatment with ISIS 7597. MRP-transfected T5 cells were exposed to MRP antisense oligonucleotide ISIS 7597 (0.5 μM) for 4 hr. MRP mRNA and protein were isolated subsequently at the times indicated on the figure, and northern and immunoblot analyses were performed. MRP mRNA levels were estimated by densitometry and normalized to the GAPDH mRNA signal; relative protein levels were also estimated by densitometry. The results shown are from a single experiment, and similar results were obtained in two additional experiments.

treatment with 0.5 μM ISIS 7597, they were only approximately 10% of those in control cells (Fig. 3), indicating that the initial decrease in MRP mRNA levels had been maintained. During the following 24 hr, MRP mRNA levels returned to 30% of controls. A similar double treatment with 0.3 μM ISIS 7597 also decreased MRP mRNA levels by 90% but for a shorter duration, and MRP mRNA had returned to 70% of control levels 48 hr after the second treatment. Levels of MRP protein following double treatment with ISIS 7597 were approximately the same whether 0.3 or 0.5 μM oligonucleotide was used (Fig. 3). MRP protein levels were decreased by 90% 24 hr after the second treatment, and this decrease was maintained for a further 24 hr. Thus, a double treatment of HeLa T5 cells with ISIS 7597 was able to extend significantly the response at the mRNA level to at least 3 days and to decrease MRP protein levels by 90%. Chemosensitivity testing at this time showed that after a 48-hr exposure to doxorubicin, the percent cytotoxicity caused by doxorubicin (10 μM) in ISIS 7597-treated T5 cells was 82 ± 9% (N = 4) compared with 50 ± 21% (N = 4) in control T5 cells ($P < 0.05$).

Cleavage of MRP mRNA by ISIS 7597

A decrease in the levels of a mRNA targeted by antisense oligonucleotides is frequently assumed to result from cleavage at the site of the RNA/DNA heteroduplex formation by members of the RNase H group of intracellular nucleases. The very rapid decrease in mRNA levels observed in our experiments with ISIS 7597 is consistent with such a mechanism. To obtain direct evidence for RNase H-mediated cleavage, RNA blots from ISIS 7597-treated HeLa T5 cells were hybridized with DNA probes corresponding to different regions of MRP mRNA to enable detection of degradative intermediates. The location of the DNA probes and the target site of ISIS 7597 on MRP mRNA are shown at the bottom of Fig. 4. Blots were first hybridized with a 5′ DNA fragment (mrp 1108) complementary to nucleotides 1219–1327, stripped, and rehybridized with a DNA probe (mrp 10.1) corresponding to the 3′ end of MRP mRNA (nucleotide 4080–5011). In addition to the expected full-length MRP mRNA species at 5.5 kb [2, 11], two smaller species were detectable when the oligonucleotide-mediated decrease was maximal (i.e. 4–24 hr after treatment) (Fig. 4). Both of these smaller species hybridize with a probe that encompasses the target sequence of ISIS 7597 (see Fig. 1). However, the 5′ DNA probe hybridized only to the smaller of the two (2.3 kb) (Fig. 4, left panel), while a 3′ probe hybridized only to the larger (3.2 kb) (Fig. 4, right panel). The sizes of these additional mRNA species (2.3 and 3.2 kb) correspond closely to the sizes of the products expected from the cleavage of T5 MRP mRNA by RNase H at the site of the ISIS 7597 target sequence.

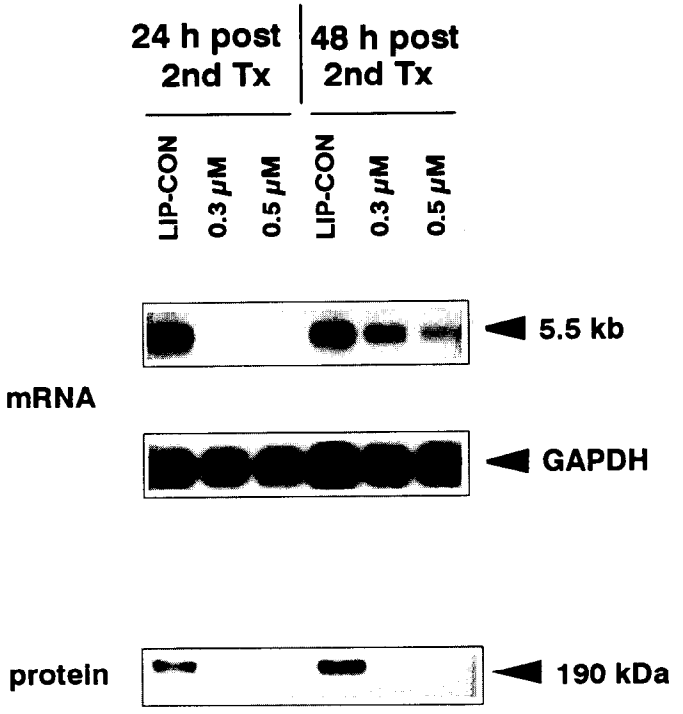

FIG. 3. Effect of a double treatment with ISIS 7597 on MRP mRNA and protein levels in transfected HeLa T5 cells. Cells were treated twice with ISIS 7597 (0.3 or 0.5 μM) complexed with lipofectin for a period of 4 hr with an interval of 48 hr between exposures. RNA and protein were isolated, and levels of MRP were quantitated 24 and 48 hr after the second treatment. The results shown are from a single experiment, and similar results were obtained in a second experiment. LIP-CON = lipofectin control.

DISCUSSION

Cells that express MRP and display a multidrug-resistant phenotype are relatively insensitive to reversing agents that are effective in circumventing resistance mediated by P-glycoprotein [11, 19–21]. The difference in sensitivity between MRP and P-glycoprotein to these agents is likely to be the result of differences in substrate specificity, particularly in view of recent data demonstrating the unique ability of MRP to transport cysteinyl leukotrienes [42, 43]. Because MRP and P-glycoprotein share only 15% amino acid identity, it is difficult to predict from structural considerations which compounds may be able to reverse MRP-mediated multidrug resistance. On the other hand, antisense oligonucleotides are able to inhibit the expression of gene products in a manner dependent on the DNA rather than the amino acid sequence (for a recent review see Wagner [44]). Consequently, the use of antisense oligonucleotides provides an attractive approach to the reversal of drug resistance because it bypasses any dependence on protein structure or function. In this study, we have evaluated antisense phosphorothioate oligonucleotides for their ability to decrease MRP expression in human cell lines.

When an antisense molecule binds to its complementary nucleic acid target, the resulting hybrid can decrease protein synthesis by a number of mechanisms. Occupancy-related mechanisms rely on the ability of the oligonucleotide to block the interaction of the RNA or DNA target with proteins or other nucleic acids required for processing or expression. Examples of this include the inhibition of RNA splicing by targeting intron/exon junctions, and translational arrest medi-

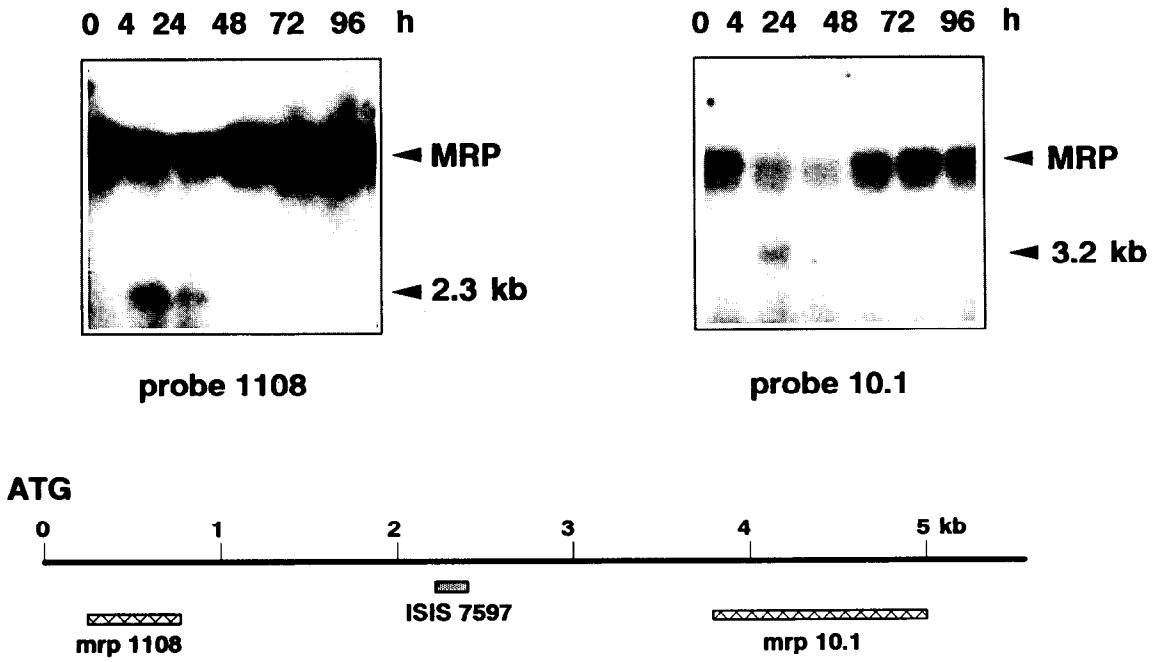

FIG. 4. Cleavage of MRP mRNA by ISIS 7597. RNA was isolated from HeLa T5 cells treated with ISIS 7597–lipofectin complexes for the times indicated, and blots were prepared as described in Materials and Methods. Blots were hybridized with a DNA fragment (mrp 1108) corresponding to a region at the 5′ end of the MRP mRNA, stripped, and rehybridized with a cDNA fragment corresponding to the 3′ end of MRP mRNA (mrp 10.1). The length of time cells were treated is indicated at the top of the blots. The position of the DNA probes and the location of the target sequence of antisense oligonucleotide ISIS 7597 (close to the first nucleotide binding domain) are shown at the bottom of the figure. In addition to the expected intact full-length MRP 5.5 kb mRNA of the transfected T5 cells, an additional band of 2.3 kb was detected with the 5′ probe, mrp 1108 (left panel), and a 3.2 kb band was detected with the 3′ probe, mrp 10.1 (right panel). The results shown are from a single experiment, and similar results were obtained in two additional experiments.

ated by oligonucleotides complementary to the region surrounding the AUG translation initiation codon. Other postulated mechanisms, termed occupancy-activated destabilization, lead to a rapid degradation of the mRNA target. One such mechanism includes the RNase H-mediated degradation of the RNA strand at the site of RNA/DNA heteroduplex formation [45–47].

Phosphorothioate oligodeoxyribonucleotides were chosen for the current study for two major reasons [48]. First, unmodified phosphodiester oligonucleotides have a limited half-life in cell culture and have an intracellular half-life that is measured in minutes rather than hours, while phosphorothioates are stable in cell culture medium and have an intracellular half-life of more than 24 hr. Second, phosphorothioates as opposed to some other modified oligonucleotides retain the ability to form a duplex with the complementary RNA sequence that is cleavable by RNase H. Digestion by RNase H is believed to be one of the predominant mechanisms involved in antisense inhibition [49, 50], although it is almost certain that other mechanisms such as hybrid arrest of translation and inhibition of RNA processing occur with some oligonucleotides [51]. After exposure of both drug-selected H69AR cells and MRP-transfected T5 cells to ISIS 7597, we observed almost complete disappearance of the MRP mRNA within 4 hr of treatment. This rapid loss of mRNA is unlikely to result solely from a hybrid arrest mechanism of inhibition, and it did not occur with the 2′-O-methyl oligonucleotide tested (ISIS 7607, Table 1). Instead, an active degradation of the MRP mRNA is suggested. Longer exposure of autoradiographs revealed low levels of two additional RNA species that were detected with MRP DNA probes in antisense-treated cells but not in controls. The sizes of these RNAs corresponded closely to the expected sizes of the cleavage products of MRP mRNA after RNase H digestion at the site of ISIS 7597 hybridization. Although it has been demonstrated that many oligonucleotides may form RNase H cleavage hybrids in crude cell lysates or when digested with purified enzyme [52, 53], direct evidence for RNase H cleavage as the mechanism of action of antisense oligonucleotides in intact cells is rare. Recently, Giles *et al.* [54] demonstrated the presence of RNase H-generated mRNA fragments in live cells after treatment with antisense oligonucleotides, but permeabilization of the cells with Streptolysin O was required. In most studies, phosphorothioate oligonucleotides have been presumed to act via RNase H-mediated cleavage because phosphorothioate 2′-O-methyl oligonucleotides with the same sequence do not inhibit expression of the gene in question [29, 53, 55]. By using two DNA probes corresponding to regions in the 5′ and 3′ coding ends of the mRNA, respectively, we were able to detect the oligonucleotide-induced cleavage fragments of MRP mRNA

in whole cells. Low levels of degradative products are to be expected since the half-lives of such intermediates are likely to be extremely short because they are unprotected at their respective extremities.

All 15 phosphorothioate deoxynucleotides were able to decrease MRP mRNA, at least to some degree. It is unlikely that the different efficacies of the oligonucleotides result from differences in uptake, because uptake of phosphorothioate oligonucleotides in the presence of cationic lipids is generally efficient, rapid, and sequence independent [56]. It has been hypothesized that differences in efficacy may be a consequence of the local secondary structure and folding of the target mRNA sequence [57]. The AUG translational start site has been targeted in many studies because of the postulated "accessibility" of this sequence. However, other regions have also been targeted effectively. For example, while Dean *et al.* [55] found oligonucleotides targeting the AUG translation initiation codon and 3′-untranslated sequences of the protein kinase Cα mRNA to be most effective, we found that oligonucleotides complementary to the coding sequence gave the greatest inhibition of MRP expression. These results indicate the importance of evaluating the activity of a number of oligonucleotides complementary to different regions of a given mRNA target rather than testing oligonucleotides directed against only a single site.

The rapid decrease in MRP mRNA levels induced by ISIS 7597 was transient and the mRNA returned to control levels within 72 hr after treatment. Although phosphorothioates are relatively stable [58], some degradation might be expected during this time period. This and the dilution effect resulting from cell division may explain the short-lived effects. It was observed that the inhibition was more prolonged in H69AR cells, which have a doubling time significantly longer than HeLa T5 cells which divide every 24 hr (data not shown). This suggests that the rapidity with which MRP mRNA returns to normal levels is influenced by the decrease in intracellular concentration of oligodeoxynucleotide during cell division.

In conclusion, we have shown that the phosphorothioate oligodeoxynucleotide ISIS 7597 specifically inhibits the expression of MRP in a concentration-, time- and sequence-dependent manner. A double treatment of transfected T5 and drug-selected H69AR cells with ISIS 7597 was required to achieve a prolonged decrease in MRP protein levels. Other investigators have obtained decreases in *MDR1* mRNA and P-glycoprotein levels with antisense oligonucleotides, but reversal of P-glycoprotein-mediated resistance has been reported to be incomplete. This was attributed to poor uptake of the oligonucleotides [59], rapid degradation of the oligonucleotide [22], or the use of an oligonucleotide with a less than optimal sequence [25]. The chemosensitivity experiments using T5 cells treated with ISIS 7597 also indicate incomplete reversal of resistance of MRP-mediated resistance, suggesting that optimal suppression of MRP expression for a more prolonged period of time may be necessary to achieve complete chemosensitizaton.

The studies described here provide strong evidence for RNase H-mediated destruction of a specific mRNA in intact cells following treatment with phosphorothioate oligodeoxynucleotides. They also demonstrate that it is possible to eliminate rapidly the expression of high levels of MRP mRNA and, with the appropriate choice of administration schedules, reduce markedly the levels of the encoded protein, despite its relatively long half-life. Clinically, chemosensitization of multidrug-resistant tumors overexpressing proteins such as MRP and P-glycoprotein is likely to be necessary for only limited periods during chemotherapy. Consequently, antisense oligonucleotides provide an attractive and potential highly specific treatment modality as an adjunct to conventional chemotherapy. Towards this end, studies in an *in vivo* model are underway to test the efficacy of phosphorothioate oligodeoxynucleotides as chemosensitizing agents in MRP expressing tumors [60].

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