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Inhibition of Expression of the Multidrug Resistance-Associated P-Glycoprotein by Phosphorothioate and 5′ Cholesterol-Conjugated Phosphorothioate Antisense Oligonucleotides

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SUMMARY

Multiple drug resistance (MDR) as a result of overexpression of the P-glycoprotein drug transporter, a product of the *MDR1* gene, is a significant problem in cancer therapeutics. We demonstrate that phosphorothioate antisense oligonucleotides can reduce levels of *MDR1* message, inhibit expression of P-glycoprotein, and affect drug uptake in MDR mouse 3T3 fibroblasts. An oligonucleotide (5995) directed against a sequence overlapping the AUG start codon was effective in reducing *MDR1* transcript and protein levels when used at submicromolar concentrations in conjunction with cationic liposomes, whereas a scrambled control oligonucleotide (10221) was ineffective. Substantial and specific antisense effects could also be attained with a 5′ cholesterol conjugate of the 5995 sequence. In this case, use of cationic liposomes was unnecessary. The 5′ cholesterol 5995, but the not 5′ cholesterol 10221, reduced *MDR1* message and P-glycoprotein levels by 50–60% when used at low micromolar concentrations. In parallel, treatment with 5′ cholesterol 5995 also enhanced cellular accumulation of rhodamine 123, a well-known substrate of the P-glycoprotein transporter. The effectiveness of the cholesterol-conjugated 5995 may be due to its rapid and extensive cell uptake, as indicated in flow cytometry and confocal microscopy studies. These observations suggest that cholesterol-conjugated antisense oligonucleotides may offer a novel approach to inhibition of P-glycoprotein-mediated MDR and to the modulation of other tumor cell genes whose overexpression contributes to the neoplastic state or to resistance to therapy.

An exciting approach for cancer chemotherapy is the potential use of antisense oligonucleotides to modulate the expression of genes responsible for malignancy or for resistance to therapy. Appropriately designed antisense oligonucleotides can influence gene expression through effects on gene stability, transcription, message processing, message stability, or translation (1–3). However, the promise of antisense therapeutics has been difficult to realize. Before antisense oligonucleotides can be used effectively in cancer therapy, a number of important issues need to be addressed, including oligonucleotide stability, problems of large-scale production, and improved specificity and potency of action (4–6). One major impediment to therapeutic use of oligonucleotides concerns the problem of efficient delivery of oligonucleotides to their sites of action in the nucleus and cytoplasm (7–9). In most cases, robust antisense effects in cell culture models have been achieved only the through use of substances, such as cationic liposomes, that enhance the delivery of oligonucleotides into cells (10, 11).

Antisense oligonucleotides can block gene expression through several distinct mechanisms, including (a) RNase H-mediated degradation of complementary mRNA (12), (b) interference with splicing of pre-mRNA (13), and (c) arrest of the translational machinery by binding to the 5′-cap site or the translation initiation region, without causing message degradation (14). Recently, several types of chemically modified oligonucleotides have been developed (4, 15, 16). Modifications on both the backbone and the ribose moiety have provided additional nuclease stability and, in some cases, greater melting temperatures for duplex formation than is the case for standard phosphodiester oligonucleotides. The chemical structure of an antisense compound also profoundly affects its potential mechanisms of action; e.g., 2′-O-methyl oligonucleotides, as well as oligonucleotides with certain

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ABBREVIATIONS: MDR, multidrug resistance (or resistant); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SSC, standard saline citrate; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ORF, open reading frame.

backbone modifications, do not permit activation of RNase H (5). In the current study, we primarily used phosphorothioate oligonucleotides (17). These combine a number of advantages, including ease of synthesis, a substantial degree of resistance to exonucleases, and the ability to activate RNase H and thus efficiently cause degradation of the RNA component of an RNA-DNA oligonucleotide duplex (3, 5, 18, 19).

A number of reports have documented the ability of conventional phosphodiester or chemically modified antisense oligonucleotides to inhibit gene expression in a sequence-specific fashion in cell culture situations. In particular, several cancer-related genes have been modulated in this way, including *ras* (20, 21), *BCR-ABL* (22), and c-*myb* (23), as well as other oncogenes. More recently, several laboratories have reported the effects of systemically administered anti-oncogene oligonucleotides in murine tumors or human tumor xenograft models (24–28). An important issue for the current report relates to the fact that most successful examples of inhibition of gene expression by antisense oligonucleotides involve messages that are expressed transiently or at relatively low levels. There are only a few examples in the literature of the successful use of antisense approaches for inhibition of expression of messages coding for abundant structural proteins or of messages from highly amplified genes (29, 30, 31). Nevertheless, it will be important, for anticancer therapy, to develop the ability to inhibit the expression of rather abundant and stable mRNAs. This might include messages coding for highly overexpressed oncogenes such as *HER-2* (31) or amplified or overexpressed genes that are involved in resistance to conventional chemotherapeutic agents (32).

Mammalian cells selected for resistance to certain antitumor drugs often display cross-resistance to other apparently unrelated drugs and thus are said to display an MDR phenotype (33). One form of MDR is based on overexpression of one or more members of a family of membrane proteins (i.e., P-glycoproteins) that serve as ATP-driven drug-efflux pumps (33–35). The human *MDR* (P-glycoprotein) gene family has two members, of which only one (*MDR1*) is responsible for resistance to cytotoxic drugs (35). Low levels of *MDR* are usually associated with increased transcription of P-glycoprotein genes, whereas high levels of *MDR* are associated with extensive gene amplification (33, 35). In highly drug-resistant cells, P-glycoprotein message and protein levels can be many times greater than in their drug-sensitive counterparts. Although *MDR* can be modulated by the use of a variety of agents that competitively inhibit P-glycoprotein-mediated antitumor drug efflux (36), several of these agents have proved to be less than ideal in clinical trials (37). Thus, the inhibition of P-glycoprotein expression using antisense oligonucleotides or other nucleic acid-based approaches seems to be an attractive alternative.

Several reports have documented a substantial impact on P-glycoprotein message and protein expression and on levels of drug resistance with the use of transfected anti-*MDR* ribozymes (38–40). As well, there are several reports of antisense oligonucleotide effects on the MDR phenotype (41–44). However, modulation of MDR using antisense approaches is a challenging undertaking; the vast overexpression of P-glycoprotein message and protein in highly resistant cells, as well as the slow turnover of P-glycoprotein (45), represents formidable difficulties for antisense technology. In this report, we used phosphorothioate oligonucleotides to demonstrate sequence-specific inhibition of the expression of P-glycoprotein message and protein in murine 3T3 cells containing an amplified human *MDR1* gene. More than 20 different antisense oligonucleotides were screened for activity. Partial inhibition of P-glycoprotein message and protein expression was attained with a phosphorothioate oligonucleotide complementary to a region flanking the AUG initiation codon. In this case, the use of cationic liposomes was essential to attain the antisense effect. In additional studies, we used a 5′ cholesterol-derivatized phosphorothioate oligonucleotide, targeted to the same sequence, to attain significant inhibition of P-glycoprotein message and protein levels, as well as partial reversal of the MDR phenotype. The 5′ cholesterol antisense compound was effective in the absence of cationic liposomes and thus may offer a more promising method of modulating MDR in both cell culture and physiological settings.

Materials and Methods

Cells. NIH 3T3 cells transfected with a plasmid containing the human *MDR1* gene (pSK1 MDR) were gift from M. M. Gottesman (46). These cells have proved to be useful models for the study of MDR phenomena. Cells were grown in DMEM medium containing 10% fetal calf serum and 60 ng/ml colchicine in an atmosphere of 95% air/5% CO_2.

Oligonucleotide synthesis. Phosphorothioate oligonucleotides and 2′-*O*-methyl phosphorothioate oligonucleotides were synthesized at ISIS Pharmaceuticals (Carlsbad, CA) using previously described procedures (19). Oligonucleotide sequences are shown in Table 1. 5′-Cholesterol-conjugated oligonucleotides were synthesized as follows. Cholesterol-3-carboxyaminohexyl-B-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite was synthesized according to the procedure reported by MacKellar *et al.* (47). First, 7.25 g of this (purified) amidite was dissolved in anhydrous dichloromethane to bring the concentration to 0.1 M. With this solution, ISIS 11073, a 5′ cholesterol-conjugated version of ISIS 5995, was synthesized by standard phosphoramidite chemistry to make an oligomer with a phosphorothioate backbone. For the cholesterol amidite coupling step, reaction time was extended to 45 min. This resulted in 85% coupling for the cholesterol amidite. After standard deprotection, the oligonucleotide-cholesterol conjugate was purified on a C-4 reverse-phase high performance liquid chromatography column (48). ISIS 12064, a 5′ cholesterol-conjugated version of ISIS 10221, was synthesized and purified in the same manner. FITC-conjugated phosphorothioate oligonucleotides with or without 3′ cholesterol conjugation were also prepared. Preparation of the fluorophore-labeled conjugates necessitated the addition of the cholesterol moiety at the 3′-end.

Treatment of cells with oligonucleotides. Cells were grown in 162-mm flasks. When 95% confluency was reached, cells were seeded onto 100-mm dishes at 5×10^6/dish in 10 ml of 10% fetal calf serum/DMEM and incubated for 24 hr. At this stage, the cells were washed two times with PBS, and then 8 ml of serum-free medium was added. For phosphorothioate oligonucleotides, 20 μg/ml Lipofectin (GIBCO BRL, Baltimore, MD) and various amounts of oligonucleotide were mixed, preincubated at room temperature for 30 min, and then incubated with the cells at 37° in a CO_2 incubator for various periods. Similar methods were used for 2′-*O*-methyl phosphorothioate oligonucleotides. For treatments with cholesterol-phosphorothioate oligonucleotides, the compounds were simply added to the cells in serum-free medium (in the absence of Lipofectin) with antibiotics and incubated at 37° in a CO_2 incubator for various periods. The cytotoxicity of the various treatments used in the oligonucleotide experiments was evaluated in preliminary experiments by using a vital dye assay. Conditions were chosen such that there

TABLE 1
Oligonucleotide sequences

Oligomer	Sequence	Target
5990	GAG CCG CTA CTC GAA TGA GC	5′ Untranslated
5993	GTT CTG GCT TCC GTT GCA CC	5′ Untranslated
5994	CCC GGC CCG GAT TGA CTG AA	5′ Untranslated
5995	CCA TCC CGA CCT CGC GCT CC	AUG codon
10440	CGG TCC CCT TCA AGA TCC AT	AUG codon
10441	CCC CTT CAA GAT CCA TCC CG	AUG codon
10442	CAA GAT CCA TCC CGA CCT CG	AUG codon
5996	CCT GGT CAT GTC TTC CTC CA	Coding (splice junction)
5997	CTT TGC CCA GAC AGC AGC TG	Coding (splice junction)
5998	GTT CAC TGG CGC TTT GTT CC	ORF and stop codon
5999	TGA ACT TGA CTG AGG AAA TG	3′ Untranslated
6002	CTT GGA AGA GCC GCT ACT CG	5′ Cap region
6003	GCC GCT ACT CGA ATG AGC GC	5′ Cap region
6004	GGA AGA GCC GCT ACT CGA AT	3′ Untranslated
6005	CTC TGT TCC TTT AAT TAC GA	3′ Untranslated
6006	TCC ACT TGA TGA TGT CTC TC	3′ Untranslated
6007	CTA TGA TTT CTC TCC ACT TG	3′ Untranslated
6010	GGC AGT CAG TTA CAG TCC AA	3′ Untranslated
6011	TTT TAG CAA GGC AGT CAG TT	3′ Untranslated
6012	TGC AAA CAT TTC AAT ACT TT	3′ Untranslated
6013	AAG TTT AGT TTT ATT ATA GA	3′ Untranslated
10221	CAC CAC CCC CCT CGC TGG TC	Scrambled control
10222	CTC CCG CAC ATC TCC GCG CC	Scrambled control
11432	GCC ACC GTC TGC CCA CTC TG	ORF
11433	GGC ACG TGC AAT GGC GAT CC	ORF
11434	CGG AGC CGC TTG GTG AGG AT	ORF
11435	AGC AGC ATC ATT GGC GAG CC	ORF
11436	CGG CCA TGG CAC CAA AGA CA	ORF
11437	TGA ACT GAC TTG CCC CAC GG	ORF
11438	GGG ATG TCC GGT CGG GTG GG	ORF
11439	TGC CCA CCA GAG CCA GCG TC	ORF
11440	ATG CCC AGG TGT GCT CGG AG	ORF
11441	GCC TCC TTT GCT GCC CTC AC	ORF
11442	TGG TGG ACA GGC GGT GAG CA	ORF
10443	2′-*O*-Methyl analog of 5995	
11073	5995 analog containing cholesterol moiety at the 5′ end	
13331	5995 analog containing cholesterol at the 3′ end and FITC at the 5′ end	
13434	5995 analog containing FITC at the 5′ end	

All entries are in the 5′-to-3′ direction.

was usually <10% difference in the number of viable cells in samples treated with oligonucleotides versus control samples maintained in medium alone; exceptions are noted in the text. The MDR-3T3 cells maintained a high level of viability during extended incubation in serum-free medium, although cell division was in large part suppressed.

Measurement of MDR1 mRNA expression by Northern blotting. Total cellular RNA was isolated by lysis in 4 M guanidium isothiocyanate followed by a cesium chloride gradient, and the RNA was resolved on 1.2% agarose gels containing 1.2% formaldehyde and transferred to nitrocellulose membranes (19). The blots were hybridized with a ^{32}P-radiolabeled human MDR1 cDNA probe. The MDR1 cDNA probe was isolated by performing a polymerase chain reaction on the pSK1 MDR plasmid, as described previously (49). The following oligonucleotide primers were used for PCR: a, GGATCTTGAAGGGGACCGCAATGGAGGAGC; and b, GTCCAACACTAAAAGCCCCAATTAATACAG. The resulting fragment was checked on an agarose gel and radiolabeled with [^{32}P]dCTP using a commercially available random primer labeling kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The filters were hybridized overnight in hybridization buffer (25 mM KPO_4, pH.7.4, 5× SSC (1× = 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, 50% formamide) (49). This was followed by two washes with 1× SSC plus 0.1% SDS and two washes with 0.25× SSC plus 0.1% SDS. Hybridizing bands were visualized by exposure to X-OMAT AR film and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To confirm equal loading of RNA, the blots were stripped and reprobed with a ^{32}P-labeled actin probe (Clontech, Palo Alto, CA).

Measurement of P-glycoprotein expression by Western blotting. Cells were seeded onto 60-mm dishes at 1.2 × 10^6/dish and incubated for 24 hr in serum-containing medium. The cells were treated with the oligomers for various times in serum-free medium as described above. Cells were then extracted in lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 500 μM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/ml aprotonin, 0.5% Triton X-100) and sonicated briefly. The lysate was spun in a microfuge tube for 20 min at 4°, and the resulting supernatant was checked for protein content. Equal amounts of protein (usually 20 μg) were mixed with SDS sample buffer and boiled. Protein samples were separated by 8% SDS-polyacrylamide gel electrophoresis, and the resolved proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked (3% bovine serum albumin and 2% nonfat dry milk in PBS) and then treated with 2 μg/ml C219 anti-P-glycoprotein antibody (Signet Laboratories, Dedham, MA). After washing three times with 0.1% Tween 20, the membranes were incubated with rabbit anti-mouse antibody (Cappel Laboratories, Durham, NC). Immunoreactive proteins were visualized either by enhanced chemiluminescence (ECL kit, Amersham, Arlington Heights, IL) or with ^{125}I-labeled secondary antibodies.

Measurement of cell surface P-glycoprotein by flow cytometry. Cells were seeded onto 60-mm dishes at 1.2 × 10^6/plate in 5 ml of medium, grown for 1 day in 10% FBS/DMEM, and exposed to the oligomers in serum-free medium. After treatment with the oligonucleotides, cells were washed twice in PBS, 0.25 ml of pancreatin was added to remove cells from the plate, and the dispersed cells were resuspended in 10% FBS/DMEM and incubated at 37° for 2 hr. After the incubation, cells were washed in PBS, and 50 μl of 20 μg/ml MRK16 anti-P-glycoprotein antibody (Kamiya, Thousand Oaks, CA) was added (50). This mixture was incubated for 45 min on ice, and the cells were washed three times in 10% FBS/PBS. Cells were then incubated for 30 min with 20 μl of secondary antibody (diluted 10-fold), an *R*-phycoerythrin-conjugated goat anti-mouse IgG (Sigma Chemical, St. Louis, MO). After the incubation, cells were washed two times in 10% FBS/PBS. Finally, the cells were resuspended in 500 μl of PBS. The level of *R*-phycoerythrin fluorescence in viable cells (as determined by light scatter) was quantified using the Cicero software application (Cytomation, Fort Collins, CO) on a Becton Dickinson (San Jose, CA) flow cytometer.

Measurement of rhodamine 123 uptake by flow cytometry. We essentially followed the procedure described by Twentyman *et al.* (51) with minor modifications. Briefly, 7.5 × 10^5 cells were seeded onto six-well plates, incubated for 1 day, and treated with oligomers

as described above. Cells were removed with pancreatin and resuspended in 10% FBS/DMEM. Rhodamine 123 (Sigma) was dissolved in water and added to a final concentration of 1.0 μg/ml; 500-μl samples were taken at several points, washed with medium once, and resuspended in 500 μl of medium. Viable cells were analyzed for the accumulation of rhodamine 123 on a Becton Dickinson flow cytometer using Cicero software.

Measurement of cellular accumulation and intracellular distribution of FITC-labeled oligonucleotides. The cellular accumulation of FITC-labeled oligonucleotides was quantified by flow cytometry. The cell uptake and intracellular distribution were visualized on a cell-by-cell basis using digitized fluorescence microscopy, essentially as we described previously (8), except that a confocal microscope system was used. Intracellular fluorescence was visualized by taking optical sections through the cell body; a section approximately half-way between the top surface of the cells and the surface of the cover slip is illustrated in each case. Phase contrast images of the same cells were also obtained. A Nikon Fluor 40/1.3 Oil Ph4DL objective was used, with Comos software controlling an MRC600 scanner/laser (BioRad, Richmond, CA). In the digitized images, gain and black level settings were optimized on cells treated with free FITC 5995 oligomer and were unchanged thereafter. Incubation conditions for the flow cytometry and confocal microscopy experiments are specified in the figure legends.

Results

Characteristics of the anti-*MDR1* oligonucleotides. A number of 20-mer phosphorothioate antisense oligonucleotides were produced and purified; these compounds were targeted to various regions of the *MDR1* message, as indicated in Fig. 1. The sequences of the oligonucleotides tested are shown in Table 1. The effects of these oligonucleotides on the MDR phenotype in 3T3 cells stably transfected with a human MDR1 cDNA were examined using several assays, including Northern blots with an *MDR1* probe, Western blots with an anti-P-glycoprotein antibody, and flow cytometric analyses to monitor surface expression of P-glycoprotein, as well as drug (rhodamine 123) accumulation. In most cases, the MDR 3T3 cells were exposed to oligonucleotides administered as a complex with cationic liposomes (Lipofectin). However, all studies with cholesterol-conjugated oligonucleotides were performed in the absence of cationic liposomes.

Identification of an antisense oligonucleotide that specifically reduces *MDR1* message expression. RNA isolated from MDR 3T3 cells was probed with a 1.0-kb PCR-based MDR1 probe; this revealed a transcript of 4.4 kb. In initial experiments, the MDR 3T3 cells were exposed to 1.0 μM concentrations of several different antisense oligonucleotides or control oligonucleotides in the presence of 20 μg/ml Lipofectin for 24 hr. One oligonucleotide, 5995, which was targeted to a region overlapping the AUG codon, caused a substantial reduction in *MDR1* message levels (Fig. 2A). Oligonucleotides 10221 and 10222 have the same base composition as 5995 but are scrambled sequences that were used as specificity controls. Northern blots were stripped and reprobed with a β-actin probe to confirm equal loading of RNA (Fig. 2B). This experiment was repeated several times, and the *MDR1* and β-actin bands on nonsaturated autoradiograms were compared by laser densitometry. The *MDR1*/actin ratios for the 5995 and 10221 oligonucleotides are shown in Fig. 2C, indicating specific inhibition of *MDR1* message levels by 5995. In additional experiments, all of the oligonucleotides listed in Table 1 were screened for their ability to reduce *MDR1* message levels when used at a 1.0 μM concentration with cationic liposomes. Only oligonucleotide 5995 consistently caused this effect. Two other oligonucleotides (10441 and 10442), which also overlapped the AUG codon but were complementary to distinct sequences (see Table 1), failed to substantially reduce *MDR1* message levels.

Time course of inhibition of *MDR1* message levels by oligonucleotide 5995. Maximum specific reduction in MDR1 mRNA was observed after 24-hr treatment of cells with 5995; longer treatment did not result in lower message levels (Fig. 3). Reduction in *MDR1* message levels was attained only when oligonucleotide treatment was performed in serum-free medium and cationic liposomes were used. This result is consistent with previous observations on antisense actions of phosphorothioate oligonucleotides in cell culture (11, 48). Multiple treatments with 5995 oligonucleotide did not cause any greater specific reduction in *MDR1* message levels than a single treatment, whereas greater cytotoxicity was observed (data not shown). The reduction in MDR1 mRNA expression was reversible because after 24-hr expo-

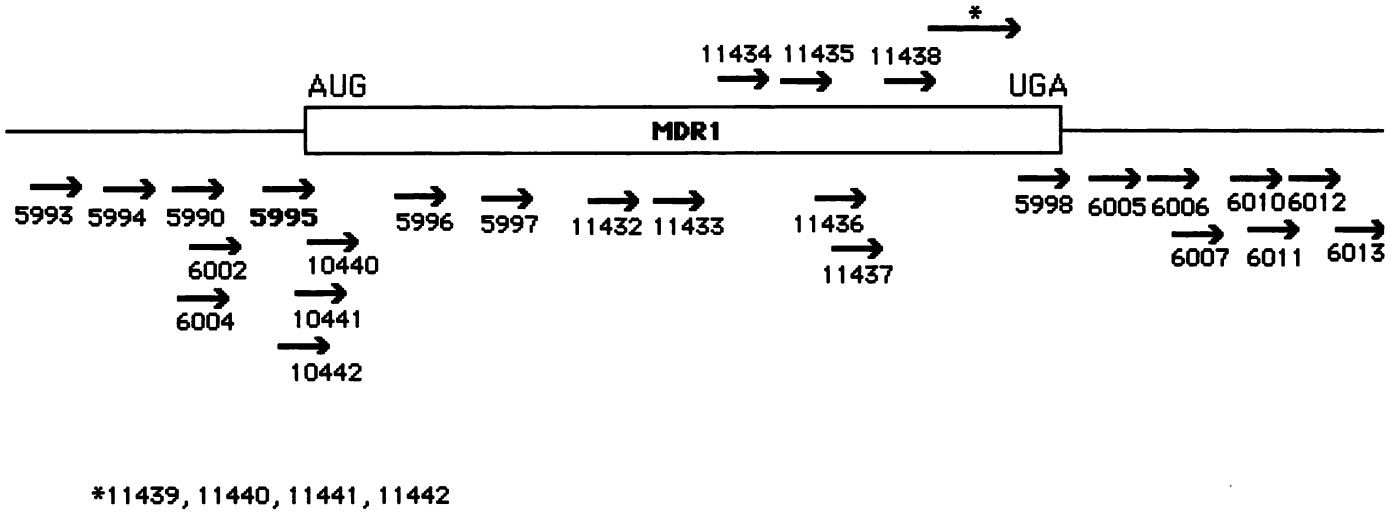

Fig. 1. Antisense targets in the *MDR1* message. The approximate sites of binding of the various antisense compounds tested are shown in the diagram. The precise sequences are given in Table 1.

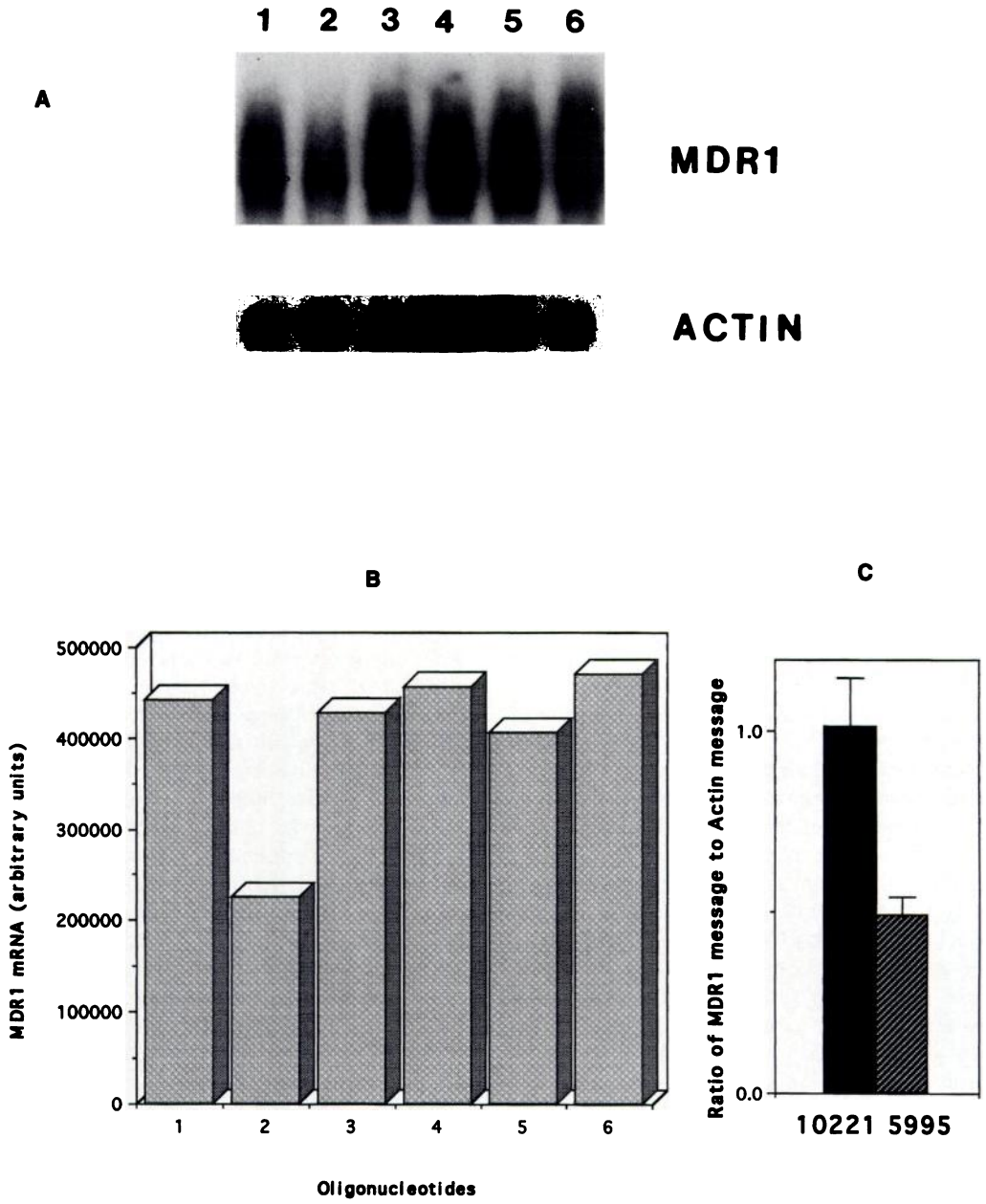

Fig. 2. Effect of different phosphorothioate oligonucleotides on *MDR1* message expression. A, NIH 3T3 cells transfected with pSK1 MDR plasmid were grown to 90% confluence and treated with 1 μM oligonucleotides for 24 hr in the presence of Lipofectin in serum-free medium. Total RNA was isolated, fractionated on agarose formaldehyde gels, and blotted onto nitrocellulose membranes, as described in Materials and Methods. These membranes were probed with a ^{32}P-radiolabeled 1.0-kb MDR1 cDNA (*top*) and then stripped and reprobed with a ^{32}P-radiolabeled β-actin cDNA (*bottom*). *Lane 1*, oligonucleotide 5990 targeted to a 5′ untranslated region. *Lane 2*, 5995 targeted to the initiation codon region. Lane 3, transfected cells incubated with Lipofectin alone. *Lanes 4* and *5*, 10221 and 10222 are scrambled control oligonucleotides for 5995. *Lane 6*, untreated MDR 3T3 cells. B, *MDR1* transcript levels were quantified using a PhosphorImager; the values are expressed in arbitrary units (the actin transcripts were essentially constant). *Numbers*, correspond to the lanes in A. C, Ratios of MDR1 and actin transcripts in cells treated with 5995 and 10221 as described in A. Results are quantified by laser densitometry and represent mean and standard error of seven independent determinations.

sure to 5995, if the cells were returned to complete culture medium, normal levels of *MDR1* message were restored within 24 hr (data not shown).

Concentration dependence of *MDR1* message reduction. Treatment of MDR 3T3 cells with oligonucleotide 5995 resulted in a concentration-dependent inhibition of *MDR1* message (Fig. 4). Some reduction in message levels was observed with concentrations as low as 100 nM. Maximal specific effects were observed at ~1.0 μM; this entailed a ~60% reduction in *MDR1* message. The use of higher concentrations of oligonucleotides (5–10 μM) with Lipofectin resulted in greater nonspecific effects, with reduced actin message levels and cytotoxicity observed with both 5995 and 10221, the scrambled control.

Role of RNase H. To determine whether the observed inhibition of P-glycoprotein expression was mediated via RNase H or by some other mechanism, we synthesized a 2′-*O*-methyl phosphorothioate analog of 5995 (oligonucleo-

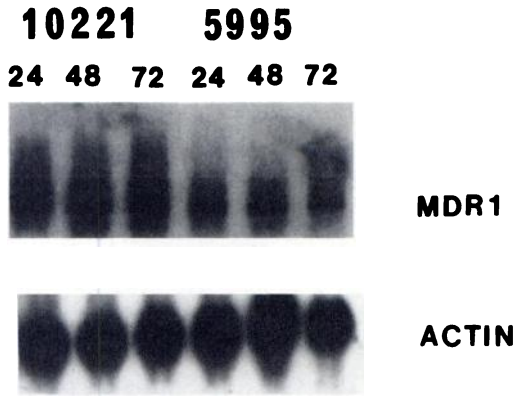

Fig. 3. Time course of inhibition of *MDR1* message. *MDR1*-transfected cells were treated with 1 μM 5995 oligonucleotide or 1 μM 10221 scrambled control oligonucleotide with Lipofectin for 24, 48, and 72 hr, respectively. RNA was isolated and separated as described above and probed with a 1.0-kb MDR1 cDNA fragment (*top*). The same blot was stripped and reprobed with a β-actin cDNA fragment (*bottom*). This experiment was repeated twice with similar results.

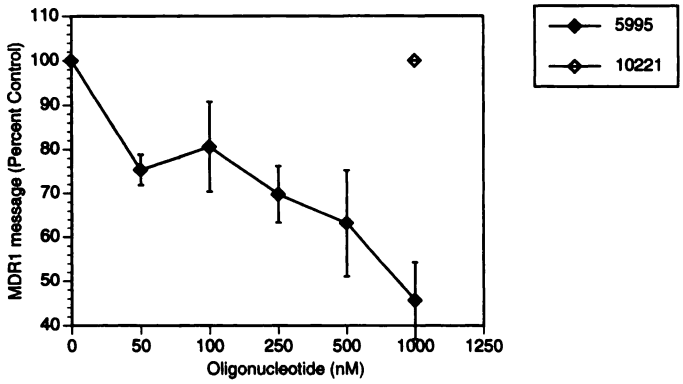

Fig. 4. Dose response for oligonucleotide inhibition of *MDR1* message expression. The MDR 3T3 cells were treated with 50, 100, 250, 500, or 1000 nM 5995 oligomer or 1000 nM 10221 scrambled control oligomer for 24 hr in the presence of Lipofectin. RNA was isolated, separated as described above, and probed with a 1.0-kb MDR1 cDNA fragment. The same blots were stripped and probed with a β-actin probe to ensure uniform loading. Levels of MDR1 mRNA from the blots were quantified using a PhosphorImager, and the values were expressed as percentage of control. Results represent mean and standard error from five independent experiments.

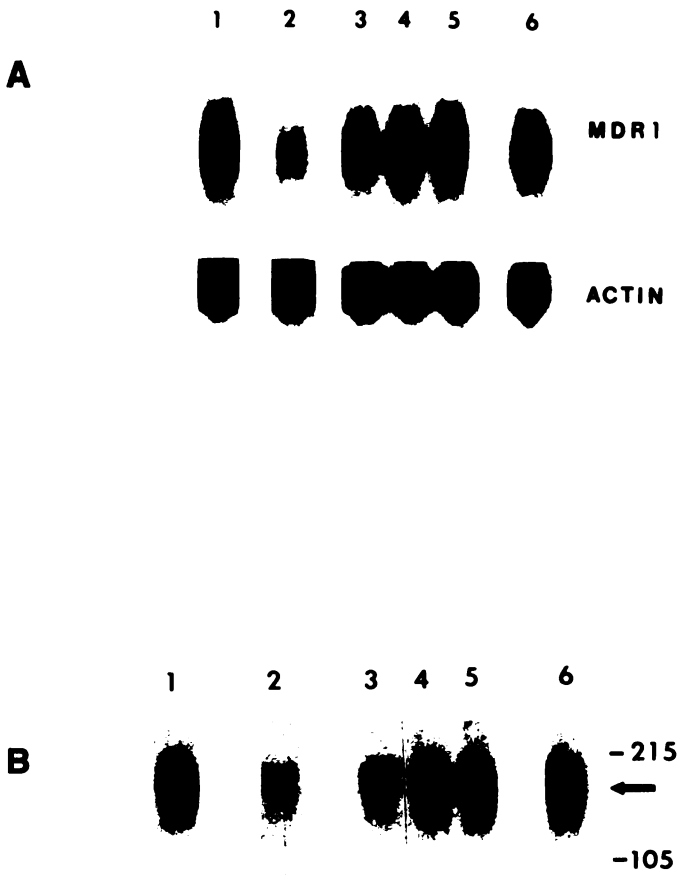

Fig. 5. Role of RNase H and of specific sequence. A, *MDR1*-transfected cells were treated with oligonucleotides 5995, 10440, 10441, 10442, or 10443 for 24 hr in the presence of Lipofectin. Total RNA was isolated and fractionated and probed with a 1.0-kb MDR1 cDNA fragment (*top*). The same blot was stripped and reprobed with a β-actin probe (*bottom*). B, *MDR1*-transfected cells were treated with oligonucleotides 5995, 10440, 10441, 10442, or 10443 for 48 hr. Cell lysates were isolated as described in Materials and Methods, normalized for total protein, and separated by 8% SDS-polyacrylamide gel electrophoresis. P-glycoprotein was detected with c219 antibody followed by enhanced chemiluminescence. *Lane 1*, 10221, scrambled control. *Lane 2*, 5995, targeted to the AUG codon region. *Lanes 3–5*, 10440, 10441, and 10442, other oligomers flanking the AUG codon region. *Lane 6*, 10443, a 2′-*O*-methyl analog of 5995. These experiments were repeated twice with similar results.

tide 10443) and performed RNA and protein analyses on cells treated with this compound. It is known that 2′-*O*-methyl oligonucleotides, although having higher Tms, are not substrates for RNase H. Treatment of MDR 3T3 cells with the 2′-*O*-methyl analog of 5995 did not reduce expression of *MDR1* message (Fig. 5A) or P-glycoprotein (Fig. 5B). These data indicate that the inhibitory effects of 5995 on P-glycoprotein expression are most likely due to RNase H-mediated message degradation than to translational arrest. We also examined other antisense oligonucleotides that overlapped the *MDR1* message start codon but were complementary to adjacent sequences (compounds 10440, 10441, and 10442; see Table 1). These had only modest effects on *MDR1* message levels, emphasizing that slight alterations in the positioning of an antisense target can be of importance in mediating antisense effects.

Inhibition of P-glycoprotein expression. The effect of 5995 antisense oligonucleotide on P-glycoprotein expression was evaluated by Western blot analysis. Consistent with the observations on message levels, expression of the P-glycoprotein was also reduced on treatment of the MDR 3T3 cells with the 5995 oligomer (Fig. 5B). Scrambled control oligomer 10221 did not reduce P-glycoprotein expression, nor did the 2′-*O*-methyl analog 10443. Other oligomers overlapping the start codon were also ineffective (compounds 10440, 10441, and 10442). The decrease in P-glycoprotein expression was minimal after 24 hr, readily detectable by 48 hr, and reached a maximum only after 72-hr exposure (not shown). Thus, effects at the protein level lag behind the observed reduction in message levels. This observation is consistent with the fact that the P-glycoprotein is quite stable and normally turns over rather slowly with a $t_{1/2}$ of 48–72 hr (52).

Effects of a cholesterol derivative of oligonucleotide 5995 on *MDR1* message levels, P-glycoprotein expression, and rhodamine 123 accumulation. The fact that we obtained only partial inhibition of *MDR1* message and P-glycoprotein expression with 5995, even after prolonged in-

cubation and after use of Lipofectin, a powerful transfecting agent, suggested that there might be insufficient or heterogeneous cellular uptake of the antisense compound. Conjugation of oligonucleotides with lipophilic substituents has been reported to enhance oligonucleotide accumulation in cells and result in improved biological effects (53, 54). For this reason, we synthesized a cholesterol derivative of 5995 (oligonucleotide 11073), as well as a cholesterol derivative of 10221 (12064), the scrambled control oligonucleotide, and examined their effects on *MDR1* message and P-glycoprotein levels. As seen in Fig. 6, treatment with concentrations of cholesterol 5995 in the range of 250 nM to 2.5 μM resulted in a specific decrease in levels of *MDR1* message. At higher concentrations (10 μM), considerably toxicity was manifest for both cholesterol 5995 and cholesterol 10221, as demonstrated by reduced vital staining of cells (not shown) and by loss of signal with the β-actin probe. It is important to note that substantial inhibition of *MDR1* message expression was attained with cholesterol 5995 without the use of cationic liposomes.

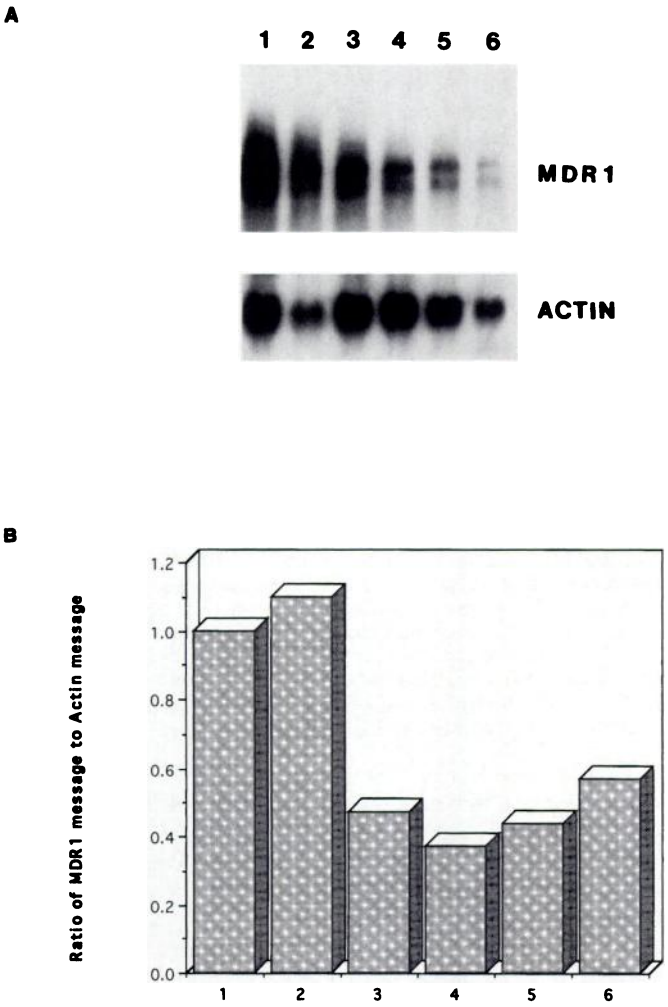

Fig. 6. Effect of cholesterol phosphorothioate oligonucleotides on *MDR1* message expression. A, MDR 3T3 cells were treated with various concentrations of oligonucleotides 5′ cholesterol 5995 or 5′ cholesterol 10221 for 24 hr in serum-free medium. Total RNA was isolated, fractionated, and probed with a 1.0-kb MDR1 cDNA fragment (top). The same blot was stripped and reprobed with a β-actin probe (*bottom*). B, The blots were quantified using a PhosphorImager and the ratio of MDR1 message to actin message was indicated. For A and B: *lane 1*, untreated MDR 3T3 cells. *Lane 2*, cells treated with 2.5 μM 5′ cholesterol 10221. *Lanes 3–6*, cells treated with 5′ cholesterol 5995 at 250 nM, 500 nM, 1.0 μM, and 2.5 μM. These experiments were repeated three times with similar results.

To observe the effects of cholesterol 5995 oligonucleotide on the expression of P-glycoprotein at the cell surface, we used immunofluorescent staining and flow cytometry, as described in above. As seen in Fig. 7A, treatment of MDR 3T3 cells with increasing concentrations cholesterol 5995 over the range of 0.5–2.5 μM resulted in a progressive reduction in surface expression of P-glycoprotein to ~40% of control levels. Some nonspecific reduction in P-glycoprotein expression was also observed with the scrambled control oligomer (cholesterol 10221), but the effect of the antisense compound was greater at all concentrations tested. In a parallel experiment, we also examined the effect on P-glycoprotein surface expression of 1 μM 5995 or 10221 administered with Lipofectin. The test concentrations of antisense or scrambled standard phosphorothioate oligonucleotides, administered with Lipofectin, were somewhat less effective than an equivalent concentration of the cholesterol-conjugated analogs. Thus, as an initial approximation, the cholesterol phosphorothioate antisense compound given alone was approximately as effective as the phosphorothioate antisense compound administered with cationic liposomes. However, in our study, the cholesterol oligonucleotides showed less experiment-to-experiment variation than did the standard phosphorothioate oligonucleotides when administered with cationic lipids.

Rhodamine 123 is fluorescent dye that is a substrate for P-glycoprotein and is rapidly transported out of MDR cells. Thus, rhodamine 123 uptake can be used as a simple and convenient way of assessing the impact of various treatments on the MDR phenotype. We evaluated the effect of antisense oligonucleotides on drug transport in MDR1 3T3 cells by monitoring rhodamine 123 using a flow cytometric assay (51). As seen in Fig. 7B, treatment of MDR 3T3 cells with increasing concentrations of cholesterol 5995 resulted in a progressive increase in rhodamine 123 accumulation, although the oligonucleotide-treated MDR 3T3 cells did not take up drug nearly as effectively as non-MDR 3T3 cells (not shown). As in the case of P-glycoprotein expression, some nonspecific effect was also observed with the scrambled control oligomer (cholesterol 10221); however, the effect of the antisense compound was significantly greater. A parallel experiment showed that standard phosphorothioate oligomers, given with cationic liposomes, also had effects on rhodamine 123 accumulation, but a lesser discrimination between antisense and scrambled sequences was observed.

Uptake and intracellular distribution of cholesterol oligonucleotides. We examined the cellular accumulation of FITC-conjugated 5995 or FITC-conjugated cholesterol 5995 using flow cytometry. As seen in Fig. 8, A and B, during a 2-hr incubation period, the cholesterol-conjugated oligonucleotide was rapidly accumulated by cells, whereas both free FITC 5995 and FITC 5995, complexed with Lipofectin, accumulated to a far lesser degree. The cellular accumulation of the cholesterol-conjugated 5995 was ~40-fold greater than that of the free 5995 at 2 hr. After overnight incubation, the free FITC 5995 still displayed significantly less cell accumulation than the FITC-cholesterol 5995; the Lipofectin-complexed FITC 5995 displayed substantial, but very heterogeneous, cell uptake after overnight incubation. Based on

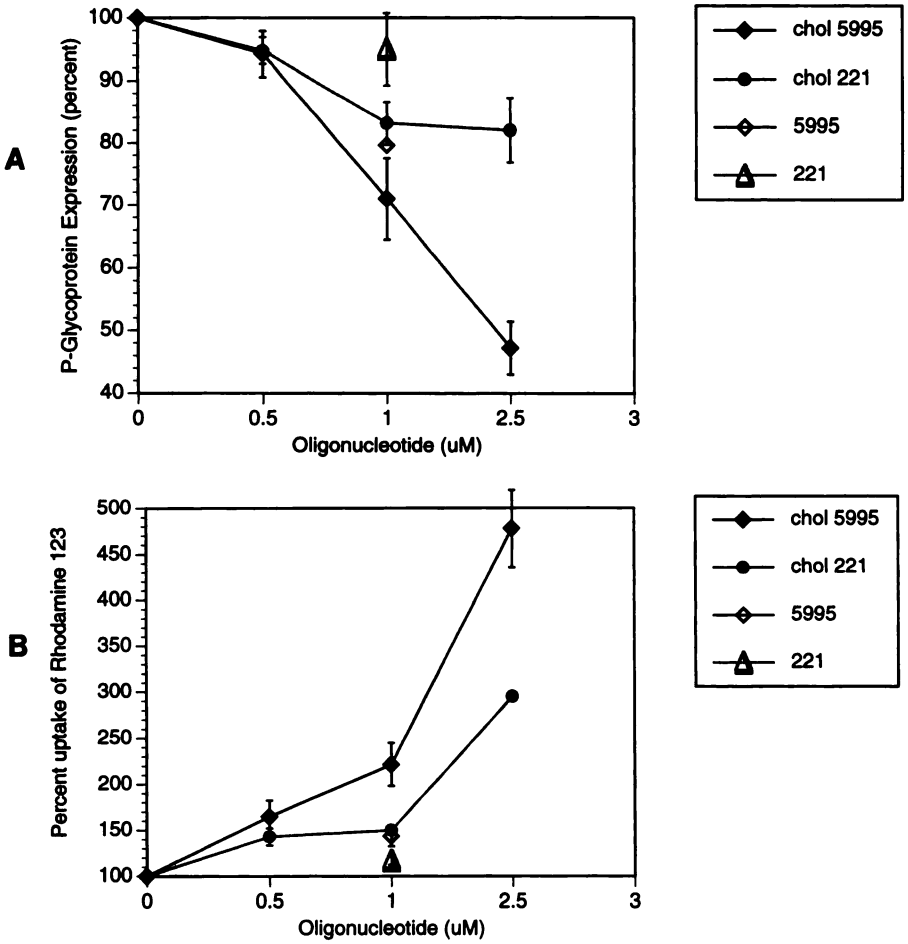

Fig. 7. Effect of cholesterol phosphorothioate oligonucleotides on P-glycoprotein surface expression and rhodamine 123 uptake. A, MDR 3T3 cells were treated with various concentrations of oligonucleotides 5′ cholesterol 5995 or 5′ cholesterol 10221 for 48 hr in serum-free medium. The cells were recovered and stained with an anti-P-glycoprotein monoclonal antibody directed against a surface epitope, followed by a phycoerythrin-conjugated second antibody. The level of cell surface fluorescence in viable cells was quantified using a flow cytometer; light scatter parameters were set so as to exclude nonviable cells. A parallel experiment was done with cells treated with oligonucleotides 5995 and 10221 at 1 μM in the presence of Lipofectin. Data are presented as percentage inhibition of P-glycoprotein expression, with the 100% level taken as that for untreated MDR 3T3 cells. The results represent mean and standard error for six determinations. B, Cells treated in a manner similar to that described in A were analyzed for rhodamine 123 uptake. Subsequent to oligonucleotide treatment, the cells were washed and then exposed to 1 μg/ml rhodamine 123 in serum-free medium at 37°. After 1 hr, the cells were washed, and the amount of rhodamine 123 accumulated by viable cells was quantified using a flow cytometer. Values are mean and standard error of three determinations.

previous experience with the stability of phosphorothioates (7), it seems likely that most of the fluorescence accumulated represents FITC oligonucleotide rather than free FITC.

Confocal microscopic images essentially confirmed and extended the flow cytometry observations. Very little cell intracellular accumulation was seen with free FITC 5995. The cells treated with FITC 5995 complexed with Lipofectin showed extensive, but very heterogeneous, cell uptake, with some cells heavily stained and others essentially blank; there also was a background of Lipofectin particles plus associated oligonucleotide bound to the coverslip. Some of the cells treated with Lipofectin showed nuclear accumulation of the fluorescence. The cells treated with 5′-FITC-3′-cholesterol oligonucleotide uniformly displayed extensive fluorescence in both the cytoplasm and nucleus. These observations suggest that the cholesterol conjugation enhanced the rapidity, amount, and uniformity of cellular uptake of the oligonucleotide and led to substantial cytoplasmic and nuclear accumulation.

Discussion

In the current study, we examined a large number of antisense oligonucleotides directed against the human *MDR1* message. These oligonucleotides were complementary to several regions of the message, including the 5′ UTR, the AUG start codon region, the coding region, and the 3′ UTR. Only one oligonucleotide sequence was found that consistently produced substantial reduction in MDR1 RNA and protein levels compared with treatment with control oligonucleotides. This sequence, 5995, was complementary to a region flanking the AUG codon; other oligonucleotides that also overlapped the AUG region but were displaced relative to 5995 failed to produce significant affects. In addition, a 2′-*O*-methyl analog of 5995, which does not support RNase H activity, failed to block *MDR1* message or P-glycoprotein expression. Our results suggest that both the ability to cause message degradation and the precise position of the antisense target in the message are important in the inhibition of P-glycoprotein expression; this agrees with recent results using antisense oligomers directed against other messages (19, 55). The identification of appropriate sequences for antisense inhibition is largely empirical at present. Clearly, both RNA secondary structure and RNA/protein association will effect the accessibility of oligonucleotides to sites on the message. As is true here, the AUG codon has often proved to be a useful target for antisense oligonucleotides. Other studies have achieved significant effects with oligonucleotides targeted to 5′ or 3′ untranslated regions (18, 19); however, that was not the case here.

We were unable to detect any antisense effects using standard phosphorothioate oligonucleotides without the use of agents designed to promote cellular uptake. In addition to Lipofectin, we tried other types of cationic lipids, as well as an amphipathic peptide (56), in an attempt to improve cell uptake of oligomers. Although the use of these other agents did result in some antisense effect (data not shown), there

was no improvement over the effects attained with Lipofectin. The requirement for Lipofectin or other uptake-enhancing agents in attaining antisense effects is consistent with previous results from a number of investigators (11, 19), although there are reports in the literature of antisense effects occurring without the use of these agents (57, 58). Treatment of cells with Lipofectin/oligonucleotide complexes entails some toxicity, especially during the rather prolonged exposures required to inhibit P-glycoprotein expression. Thus, it is extremely important to use the appropriate controls to discriminate between true antisense actions and effects that are due to toxicity of Lipofectin and/or oligonucleotide. In the current study, we obtained partial (>50%) inhibition of *MDR1* message and P-glycoprotein expression using phosphorothioate antisense oligonucleotides. Increasing the concentration of the oligonucleotides beyond 1 μM or increasing the duration of exposure did not enhance specific antisense effects but rather caused nonspecific toxicity. Robust antisense effects were also attained with a 5′ cholesterol-conjugated version of 5995, without the use of cationic liposomes. This compound, when used in the range of 250 nM to 2.5 μM, produced significant (50–60%) and specific reduction in MDR1 message levels and P-glycoprotein expression, as well as enhanced accumulation of rhodamine 123 in the MDR 3T3 cells. The basis of the dramatically more potent effects of the cholesterol-conjugated compound compared with its standard phosphorothioate homolog (when used in the absence of cationic lipids) is not entirely clear. Cholesterol conjugation has been reported to enhance oligonucleotide uptake (53, 54). Our studies indicate that this chemical modification did indeed increase the rate, extent, and uniformity of oligonucleotide uptake and seemed to afford substantial accumulation in the cytoplasm and nucleus. However, subtle aspects of oligonucleotide subcellular distribution and/or binding to intracellular components may also affect the potency of cholesterol phosphorothioates compared with standard phosphorothioate oligonucleotides.

Other investigators have previously used ribozyme and antisense approaches to modulate MDR. Substantial inhibition of P-glycoprotein expression has been obtained with anti-*MDR1* ribozymes or ribozymes directed against c-*fos*, which regulates expression of the *MDR1* message (38–40). However, in these cases, the ribozymes were transfected, clones were selected that had substantial levels of ribozyme expression, and *MDR1* expression was examined in the selected clones. This is quite a different strategy than is used in antisense studies, in which the entire cell population is evaluated. There have also been previous studies of the action of antisense oligonucleotides on MDR tumor cells (41–44). For example, Rivoltini *et al.* (42) treated doxorubicin-resistant adenocarcinoma cells with phosphodiester oligonucleotides directed against the *MDR1* initiation codon. The concentrations used were in the 10 mM range, and the oligonucleotides were used in the absence of Lipofectin for several days. Although changes in drug resistance and in P-glycoprotein expression (as measured by flow cytometry) were claimed, extremely high concentrations of oligonucleotide were required, and supporting biochemical evidence for specific antisense effects was lacking. In a later study, Corrias and Tonini (43) used several different phosphodiester oligonucleotides to inhibit MDR in human adenocarcinoma cells. In this report, partial inhibition of *MDR1* message and of P-glycoprotein was observed subsequent to treatment with 30 μM antisense oligonucleotides. The magnitude of the effects seen in this study seem similar to the ones we have observed. However, in contrast to the results of Corrias and Tonini (43), we have never observed specific antisense effects in this system or other systems with unmodified phosphodiester oligonucleotides.

The current study clearly demonstrates specific antisense inhibition of *MDR1* message and P-glycoprotein expression. The key parameters that seem to affect the magnitude of the inhibition are the target site of the antisense compound, its ability to activate RNase H, and the degree and uniformity of

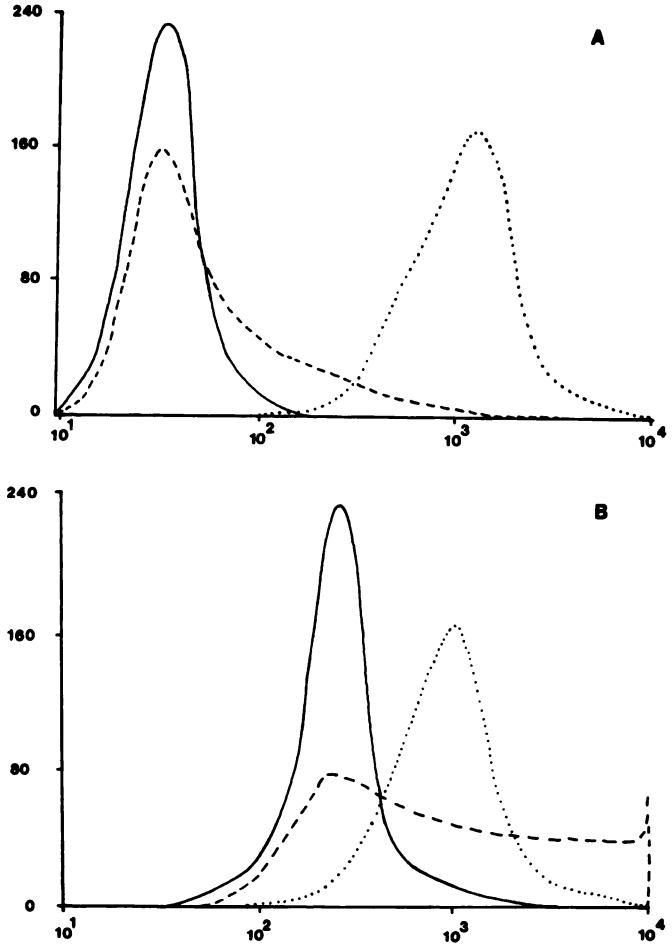

Fig. 8. Uptake and intracellular distribution of cholesterol oligonucleotides. MDR 3T3 cells were treated with 1 μM 5′ FITC 3′ cholesterol 5995 or with 1 μM 5′ FITC 5995 (with or without Lipofectin) for 2 (A) or 18 (B) hr in serum-free medium at 37°. Cells were harvested, and the fluorescence profiles were determined using a flow cytometer; light scatter parameters were set so as to exclude nonviable cells. In the case of the 18-hr treatment with Lipofectin, there were some cells with very high levels (>10^4 units) of fluorescence; these were accumulated in one channel (*right, vertical line*). *Solid line*, free 5′ FITC 5995. *Dashed line*, 5′ FITC 5995 plus Lipofectin. *Dotted line*, 5′ FITC 3′ cholesterol 5995. C–E, Cells plated onto glass coverslips were treated as above for 18 hr and then examined by confocal microscopy as described in Materials and Methods. *Left*, fluorescent images. *Right*, corresponding phase images. C, Free 5′ FITC 5995 (5 μM), D, 5′ FITC 5995 plus Lipofectin (1 μM). E, 5′ FITC 3′ cholesterol 5995 (1 μM). It should be noted that these studies were done with 5′ FITC 3′ cholesterol oligonucleotides rather than the 5′ cholesterol oligonucleotides used for experiments represented by Fig. 7. However, preliminary experiments have indicated that 3′ cholesterol 5995 has the same effect on P-glycoprotein expression as 5′ cholesterol 5995 (not shown).

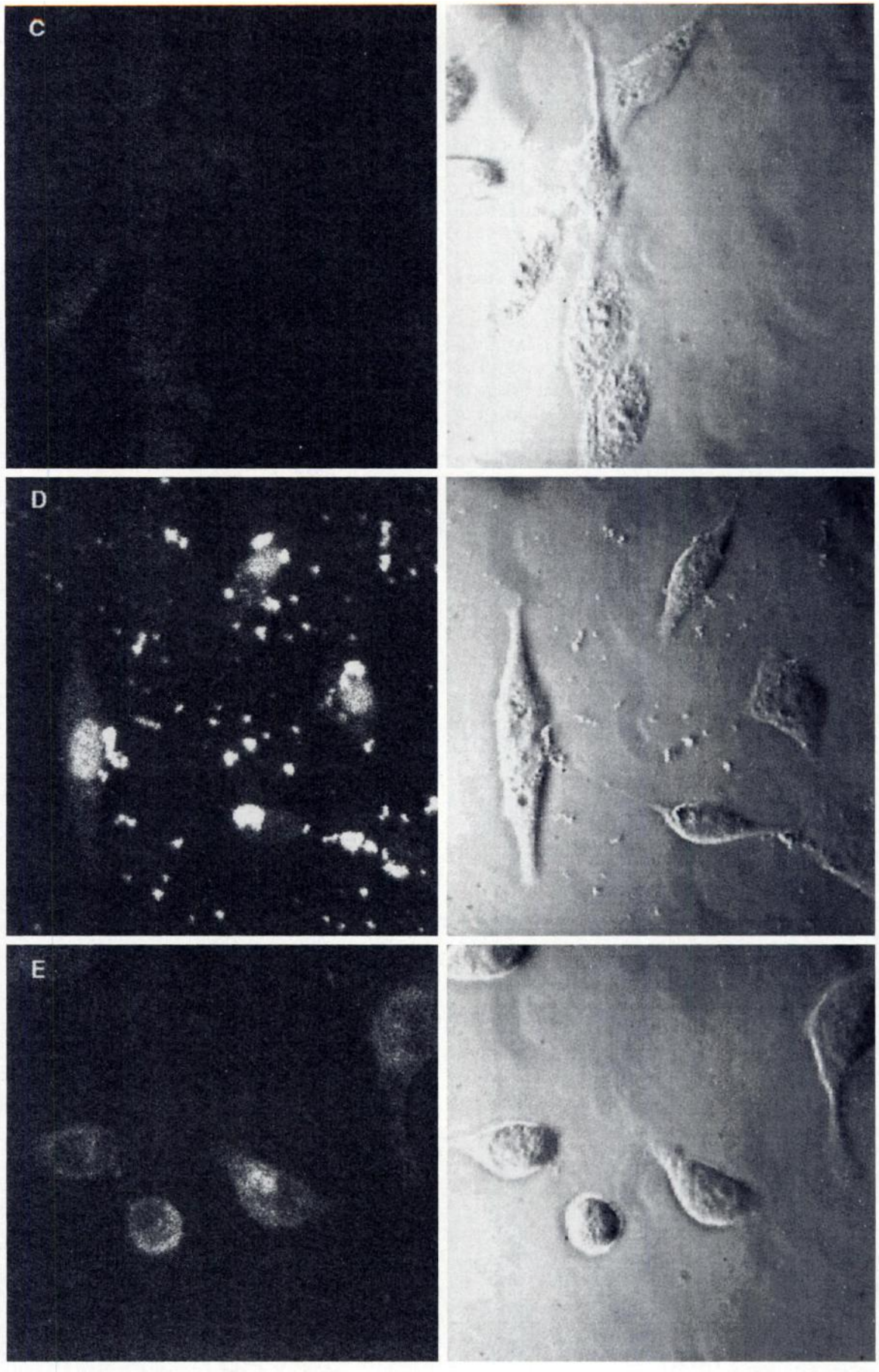

Fig. 8, C–E.

cellular uptake. Phosphorothioate oligonucleotides, used with cationic lipids, gave partial reduction of *MDR1* message and P-glycoprotein, whereas only modest effects on drug accumulation were observed. A cholesterol-derivatized phosphorothioate oligonucleotide produced substantial (50–60%) and specific inhibition of *MDR1* message and protein expression and had an effect on drug accumulation, without the need for cationic lipids. Furthermore, our experience suggests that the cholesterol-conjugated oligonucleotides may be more reliable than standard oligonucleotides complexed with cationic lipids in producing antisense effects; there seemed to be considerably greater uniformity of cell uptake and less interexperiment variation with the use of the cholesterol oligonucleotides.

Clearly, the results that we attained with a partial reduction in P-glycoprotein expression represent only an initial step toward effective control of MDR through the use of antisense approaches. Additional chemical modifications of the backbone and ribose ring structures may be needed to generate antisense compounds with greater affinity for *MDR1* message, as well as even greater stability in cells. An important finding from the current study is that there may be significant advantages in using relatively low-molecular-weight cholesterol oligonucleotides compared with extremely large oligonucleotide/cationic lipid complexes (10). This might have an significant impact in terms of pharmacokinetics, biodistribution, and tissue penetration. It will be important to determine whether the *in vitro* results described here can be extrapolated to modulation of P-glycoprotein expression and function in drug-resistant tumors *in vivo*.

Acknowledgments

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