In Vitro and in Vivo Reversal of Multidrug Resistance in a Human Leukemia-resistant Cell Line by mdrl Antisense Oligodeoxynucleotides

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Abstract

A major obstacle to successful cancer chemotherapy is the development of multidrug resistance (MDR) by tumor cells. Overexpression of the mdrl gene product P-glycoprotein (P-170) is characteristic of such cells. In this study, in vitro and in vivo reversion of MDR was attempted in a human leukemia cell line resistant to vincristine (HL-60/Vinc) using an 18-mer mdrl antisense phosphorothioate oligodeoxynucleotide ([S]ODN) in combination with vincristine. As control of sequence specificity, both sense and scrambled [S]ODNs were used. The ability of these [S]ODNs to reverse MDR was studied in vitro and in severe combined immunodeficient (SCID) mice. In vitro treatment with antisense [S]ODNs restored vincristine sensitivity of HL-60/A'inc cells, whereas no changes in drug sensitivity were observed upon treatment with the sense or scrambled sequence. The in vitro effects correlated with inhibition of P-170 expression in HL-60/Vinc cells exposed to the mdrl antisense [S]ODNs. In vivo reversal of MDR was obtained in SCID mice given injections of HL-60/Vinc cells and systemically treated with [S]ODNs plus vincristine, as indicated by a significantly prolonged survival of SCID mice that received the combination therapy of mdrl antisense [S]ODNs + vincristine. Treatments with mdrl antisense or scrambled [S]ODNs, vincristine, or scrambled [S]ODNs + vincristine had no effect on survival. These results suggest that the use of mdrl antisense ODNs in combination with standard antineoplastic drugs might be useful in reversing MDR in vitro and in vivo.

Introduction

The resistance of tumor cells to multiple chemotherapeutic agents, a phenomenon termed MDR, is a major obstacle to successful cancer chemotherapy and has been closely associated with treatment failure. MDR is characterized by cross-resistance to a number of structurally and functionally unrelated drugs, due to overexpression of the mdrl gene product P-glycoprotein. Other mechanisms such as overexpression of glutathione S-transferase or mutation of topoisomerase II may also play a role in MDR. P-glycoprotein is a 170 KDa membrane glycoprotein that acts as an ATP-dependent efflux pump, increasing transport of various anticancer compounds out of cells and decreasing cellular accumulation of drugs and, thus, their efficacy. Anticancer drugs that are associated with P-170-mediated drug resistance include Vinca alkaloids (vinblastine and vincristine), anthracyclines (Adriamycin), Taxol, actinomycin D, and mitomycin C. Since there is a well-established correlation between the expression of the mdrl gene and the activity of the P-170-mediated transport mechanism in human tumors, compounds that can inhibit efflux by P-glycoprotein and enhance the accumulation of anticancer drugs might prove to be therapeutically useful. A variety of pharmacological agents, including verapamil, other calcium channel blockers, calmodulin inhibitors, cyclosporins, and steroid hormones, have been shown to interfere with P-glycoprotein function and to successfully reverse the MDR phenotype in vitro. However, the efficacy of these agents in animal studies and clinical trials has been disappointing due to dose-limiting toxicity and lack of specificity. Consequently, much effort is currently being directed toward developing compounds that inhibit P-170, reverse the MDR phenotype, and sensitize tumor cells to conventional chemotherapeutic agents without undesired toxic effects.

AS ODNs have attracted considerable interest as a tool to inhibit gene expression (6–8), raising the possibility that such compounds might be used to inhibit P-170 expression (9, 10). Although AS ODN-mediated modulation of MDR in cultured cells has been described by several groups (9–11), to date, no clear example of reversal of MDR by ODNs has been reported in vivo.

In the present study, using a nuclease-resistant 18-mer AS [S]ODNs in combination with VINC, we were able to inhibit P-glycoprotein expression and to reverse MDR in a human leukemia cell line resistant to VINC (HL-60/Vinc) in vitro and in SCID mice systemically injected with the leukemia cells.

Thus, these studies raise the possibility that AS ODNs targeting of mdrl mRNA might be useful to restore drug sensitivity in cancer patients developing MDR-mediated drug resistance.

Materials and Methods

Cell Line and Culture Conditions

The HL-60/Vinc human promyelocytic cell line isolated from parental HL-60 cells for resistance to VINC (13) was kindly provided by Dr. Melvin Center (Kansas State University, Manhattan, KS). Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 102 μg/ml streptomycin, 102 μg/ml penicillin G, and 120 μg/ml L-glutamine in a humid 5% CO₂ incubator at 37°C. Cells were grown to a density of 1 × 10⁵ cells/ml before subculturing and were routinely tested for Mycoplasma contamination using a commercial kit (Boehringer Mannheim GmbH, Mannheim, Germany). HL-60/Vinc cells were not maintained in the presence of VINC because mdrl is constitutively overexpressed as confirmed by routinely checking mdrl mRNA and P-170 glycoprotein levels. However, fresh aliquots of HL-60/Vinc cells and HL-60 parental cells (as negative control) were defrosted from liquid nitrogen stocks every 2 months during the study and analyzed for mdrl expression and drug response before use in each experiment.

Drugs and ODNs

VINC (Oncovin; Eli Lilly, Indianapolis, IN), as supplied for clinical use, was dissolved in double-distilled water and diluted further in HBSS (Life Technologies, Inc., Grand Island, NY) to obtain the desired final concentrations. VINC stock solutions were made up fresh before each experiment.
[S]ODNs were synthesized on an Applied Biosystems (Foster City, CA) DNA automated synthesizer model 380B and were kindly provided by Lynx Therapeutics, Inc. (Hayward, CA). The sequences of the mdr1 AS and sense [S]ODNs are 5'-GTCCCCCTCAAGATCCAT-3' and 5'-ATGGATCTTGAAGGGGAC-3', respectively. They are complementary (or corresponding) to codons 1–6 of the published human mdr1 cDNA sequence (14). As control of sequence specificity, an 18-mer [S]ODN with base content identical to the AS sequence, but in SCR order, was also used. [S]ODNs were resuspended (10 mg/ml) in sterile HBSS and stored at −20°C in small aliquots.

**In Vitro [S]ODNs and Drug Treatment**

To assess whether individually used mdr1 [S]ODNs had any effect on cell proliferation, 2 × 10⁴ HL-60/Vinc cells were seeded in 24-well plates (Costar Corp., Cambridge, MA) in complete RPMI 1640 and treated with mdr1 sense, AS, or SCR [S]ODNs at a total dose of 200 μg/ml over 4 days (80 μg/ml at day 0 and 40 μg/ml from days 1 to 3) or at a total dose of 360 μg/ml over 8 days (80 μg/ml at day 0 and 40 μg/ml from days 1 to 7). Control cells were grown in the same conditions without [S]ODNs. Cell counts and viability (trypan blue dye exclusion) were determined daily until the eighth day of culture on quadruplicate samples from each treatment.

To assess the antiproliferative effects of the [S]ODNs/drug combination, 2 × 10⁴ HL-60/Vinc cells were seeded in 24-well plates and treated with sense, AS, or SCR mdr1 [S]ODNs at a total dose of 200 μg/ml, as described above. [S]ODN-treated cells were then exposed to VINC at doses from 0.01 to 1 μg/ml for 72 h. Control cells were grown in the same conditions without [S]ODNs and drug. At the end of the combination treatment, quadruplicate samples from each group were harvested, counted, and assayed for viability (trypan blue dye exclusion).

HL-60/Vinc cells (2 × 10⁴), seeded in 24-well plates, were treated with mdr1 [S]ODNs and VINC as described above. At the end of the treatment, cell suspension aliquots from control and treated groups were plated in semisolid methylcellulose medium HCC 4230 (Stem Cell Technologies, Inc., Vancouver, British Columbia, Canada) in triplicate 35-mm Petri dishes. Colony formation was scored on days 10–12. The surviving fractions were calculated by dividing the absolute survival of each treated sample by the absolute survival of the control sample.

**Western Blotting**

HL-60/Vinc cells (1 × 10⁵) were plated in 6-well plates (Costar) and treated with mdr1 sense, AS, or SCR [S]ODNs at a total dose of 200 μg/ml over 4 days as described above. At the end of [S]ODN treatment, cells were collected, counted, assayed for viability, and washed twice with calcium- and magnesium-free cold PBS. Cells (1 × 10⁶) from each sample were solubilized in cell lysis buffer [50 mM Hepes (pH 7.0), 500 mM NaCl, 1% NP40, 10 μg/ml each aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₄, and 1 mM EDTA] kept on ice for 30 min, frozen at −80°C, and thawed on ice. Cell lysates were centrifuged at 13,600 × g at 4°C for 15 min, and postnuclear supernatants were collected. Equal amounts of protein lysates (50 μg) were separated on 7% SDS-polyacrylamide gels and transferred to nitrocellulose (Trans-Blot Transfer Medium; Bio-Rad Laboratories, Richmond, CA) in glycine transfer buffer [192 mM glycine, 25 mM Tris (pH 8.8), and 20% v/v methanol]. Filters were saturated with 5% nonfat dry milk in TBS containing 0.5% Tween 20 and then incubated (12 h at 4°C) with the C219 anti-P-70 monoclonal antibody (Signet Laboratories, Inc., Dedham, MA) at a concentration of 0.4 μg/ml in TBS containing 2% nonfat dry milk and 0.05% Tween 20. Filters were washed five times with 0.25% Tween 20/0.25% NP40 in TBS and incubated with sheep anti-mouse IgG linked to horseradish peroxidase (Amersham Corp., Arlington Heights, IL) at a 1:10,000 dilution in 2% nonfat dry milk in TBS (1 h at room temperature). Bound proteins were detected using the ECL Western blotting detection system (Amersham Corp.) according to the manufacturer’s instructions. To assess protein amounts loaded and transferred onto nitrocellulose membranes, filters were stripped from the P-170 antibody and blotted with murine anti-human HSP (HSP 72/73; Oncogene Science, Inc., Uniondale, NJ). As another control of [S]ODNs specificity for P-170 protein, after stripping, filters were incubated with an anti-myb monoclonal antibody (UBI 05-175; Upstate Biotechnology, Inc., Lake Placid, NY). Both HSP 72/73 and c-myc antibody were used at 1:1000 dilutions.

**RNA Extraction and RT-PCR Analysis**

Total RNA was extracted from cells using the acid guanidinium thiocyanate-phenol-chloroform technique (15). mdr1 and β-actin mRNA transcripts were detected using RT-PCR as described (16), with minor modifications. mdr1 expression was detected with 3’ and 5’ primers corresponding to nucleotides 535–556 and 361–381, respectively, of the published cDNA sequence (14). Using these primers, PCR yields a 196-bp product (17). Evaluation of β-actin expression, used as control of the RNA amount, was carried out by using 3’ and 5’ primers corresponding to nucleotides 885–905 and 600–621, respectively, which yield a 306-bp product.

**RT Reaction.** RNA from each sample was divided into two aliquots (0.5 μg each) that were separately reverse transcribed using 400 units of Moloney murine leukemia virus reverse transcriptase (Mo-MLV-RT; Life Technologies, Inc., Gaithersburg, MD) and 100 ng of mdr1 or β-actin 3’ primer for 1 h at 37°C.

**PCR.** The resulting cDNA fragments were amplified with 5 units of Taq polymerase (Boehringer Mannheim) and 200 ng of mdr1 or β-actin 3’ and 5’ primers in a GeneAmp PCR System 9600 (Perkin Elmer-Cetus, Norwalk, CT) for 35 cycles of sequential denaturation (at 94°C for 45 s), annealing (at 55°C for 45 s), and extension (at 72°C for 30 s). After the last cycle, all PCR products were subjected to a final extension of 10 min at 72°C. After PCR, 20 μl of amplification products were electrophoresed on 2% agarose gels, transferred to Zeta-probe membranes (Bio-Rad Laboratories), and hybridized overnight at 49°C using 5’ end-labeled [3²P]ATP oligonucleotide probes. β-actin and mdr1 PCR transcripts were detected with probes corresponding to nucleotides 795–815 and 478–501, respectively. After hybridization, filters were washed in 2 × SSC and 0.1% SDS at 50°C and exposed to X-ray films at −80°C.

**Mice, Leukemia Cell Inoculation, and in Vivo Treatment with mdr1 [S]ODNs and VINC**

SCID mice were purchased from Taconic Farms (Germantown, NY) and maintained under sterile conditions. SCID mice are excellent hosts for human hematopoietic cells because defective T- and B-lymphocyte development makes these animals severely immunocompromised. Nevertheless, the presence of residual immune cells usually requires that these animals be irradiated prior to engrafting of human hematopoietic tissue (12, 18). Male SCID mice (6–8 weeks old) were irradiated (200 cGy total body irradiation), and the following day (day 1) were given i.v. injections of 5 × 10⁶ HL-60/Vinc cells in 0.2 ml of RPMI 1640. [S]ODNs (AS or SCR) were administered i.v., starting 7 days after inoculation of leukemia cells, at a dose of 1 mg/mouse/day for 10 consecutive days (days 8–17). VINC treatment was started 10 days after inoculation of leukemia cells by i.v. injection of 10% of the previously calculated lethal dose (LD₅₀) subdivided into three injections of 20 μg/mouse/day (days 11, 14, and 17). The following schedules were used: (a) mdr1 AS [S]ODNs for 10 days; (b) mdr1 SCR [S]ODNs for 10 days; (c) VINC for 3 days; (d) mdr1 AS [S]ODNs for 10 days plus VINC for 3 days; and (e) mdr1 SCR [S]ODNs for 10 days plus VINC for 3 days. Mice in the control group received 0.2 ml of HBSS i.v. for 10 days. Each experimental group consisted of 10 mice.

The effect of the different treatments was evaluated in terms of survival of HL-60/Vinc-injected mice. Survival times were calculated from the day of leukemia cell injection. Statistical significance of survival differences among animals in the various treatment groups was assessed using the Mann-Whitney U nonparametric method. P values <0.01 were considered highly significant; P values <0.05 were judged to be of statistical significance; and P values >0.05 were considered not significant.

**Results and Discussion**

**Effect of mdr1 [S]ODNs on HL-60/Vinc Cell Proliferation.** We first assessed the effects of mdr1 [S]ODNs in *in vitro* HL-60/Vinc cell proliferation (Fig. 1). As indicated by the growth curves of HL-60/Vinc cells exposed to mdr1 sense or AS [S]ODNs at a total dose of 200 μg/ml over 4 days (A) and by the growth curves of HL-60/Vinc cells treated with mdr1 SCR and AS [S]ODNs at a total dose of 360 μg/ml over 8 days (B), neither treatment had an effect on
REVERSAL OF MDR BY ANTISENSE ODNs

In Vitro Reversal of MDR in HL-60/Vinc Cells Treated with mdr1 AS [S]ODNs and Vinc. The ability of mdr1 AS [S]ODNs to increase VINC cytotoxicity in HL-60/Vinc-resistant cells and thus to reverse MDR resistance was assessed in combination experiments in which HL-60/Vinc cells were first exposed to mdr1 AS [S]ODNs and then to VINC (see “Materials and Methods”; Fig. 2A). Treatment with VINC alone had modest effects on HL-60/Vinc cell proliferation; only at the highest dose (1 μg/ml) did the inhibition of cell proliferation, expressed as percentage of the untreated control, reach 48%. Of the three combination treatments including mdr1 [S]ODNs and VINC, only the exposure to AS [S]ODNs followed by VINC significantly increased VINC cytotoxicity: the inhibition of cell growth was ~58% at the lowest VINC dose used (0.01 μg/ml) and increased up to ~92% at the highest VINC dose (1 μg/ml). In contrast, no significant effect was observed when VINC was combined with sense or SCR [S]ODNs. The in vitro restoration of VINC sensitivity in HL-60/Vinc cells was confirmed by the VINC IC50 value (VINC dose that caused 50% of growth inhibition) for each treatment. IC50 values were calculated form the percentage of growth inhibition caused by the different treatments on HL-60/Vinc cells. HL-60 parental cells and HL-60/Vinc cells showed a marked difference in the IC50 value following exposure to VINC (0.008 and 0.95 μg/ml, respectively), with a resistance index of about 120. Treatment of HL-60/Vinc cells with mdr1 sense or SCR [S]ODNs followed by VINC did not signif-

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 1. Effect of mdr1 [S]ODNs on HL-60/Vinc cell proliferation. In A, cells were treated with sense (■) or AS (□) mdr1 [S]ODNs at a total dose of 200 μg/ml over 4 days (80 μg/ml at day 0 and 40 μg/ml from days 1 to 3). Control cells (▲) were left untreated. Cell counts and viability were determined daily until the eighth day of culture. Representative of three different experiments with similar results. In B, cells were treated with SCR (■) or AS (□) mdr1 [S]ODNs at a total dose of 360 μg/ml over 8 days (80 μg/ml at day 0 and 40 μg/ml from days 1 to 7). Control cells (▲) were left untreated. Cell counts and viability were determined daily until the eighth day of culture. Representative of three different experiments with similar results. In both A and B, each value is an average of four different determinations within the same experiment. When not shown, the SE is smaller than the symbol. Bars, SE.

![Graph A](image3.png)

![Graph B](image4.png)

Fig. 2. Effect of mdr1 [S]ODNs + VINC on HL-60/Vinc cell proliferation (A) and cell survival (B). In A, cells were treated with mdr1 sense (□), SCR (●), or AS (■) [S]ODNs at a total dose of 200 μg/ml fractioned in 4 days (80 μg/ml at day 0 and 40 μg/ml from days 1 to 3) and then exposed to VINC (0.01–1 μg/ml) for 72 h. VINC-treated cells (■) were exposed to VINC alone (same doses and exposure time). Control cells were left untreated (data not shown). Representative of four different experiments with similar results. Each value is an average of four different determinations within the same experiment. In B, cells were treated with mdr1 [S]ODNs + VINC or with VINC alone as described in A. Control cells were left untreated. At the end of the treatments, methylcellulose clonogenic assays were performed. Colonies were scored 10–12 days after plating, and the surviving fractions were calculated by dividing the absolute survival of the treated samples by that of the control samples. ▲, VINC; ○, sense + VINC; ●, SCR + VINC; and ■, AS + VINC. Representative of three different experiments with similar results. Each value is an average of three different determinations within the same experiment. When not shown, the SE is smaller than the symbol. Bars, SE.
an anti-gp-170 monoclonal antibody. As a control of protein amounts loaded and blotted and of S]ODN specificity for gp-170 protein down-regulation. HSP 72/73 and MYB protein

2). SCR (Lane 3). or AS (Lane 4) S]ODN (as described in the legend to Fig. 2). gp-170 levels were assessed with Western blot performed at the end of mdrl [S]ODN treatment using mRNA, we evaluated mdrl mRNA expression in HL-60/Vinc cells

antiproliferative effect of the various compounds with levels of mdrl mRNA, we evaluated mdrl mRNA expression in HL-60/Vinc cells treated with mdrl [S]ODNs. In A, cells were exposed to mdrl sense or AS [S]ODNs (as reported in the legend to Fig. 2). 0.5 μg/ml VINC (for 72 h), and mdrl sense or AS [S]ODNs followed by VINC. Control cells were left untreated. To assess the mdrl mRNA level, cells were harvested at the end of treatment, and total RNA was isolated and divided into two equal portions and separately amplified using RT-PCR (35 cycles) with mdrl and β-actin-specific primers. Lane 1. untreated cells; Lane 2. VIN-treated cells; Lane 3. mdrl sense [S]ODN-treated cells; Lane 4. mdrl AS [S]ODN-treated cells; Lane 5. mdrl sense [S]ODNs + VIN-treated cells; and Lane 6. mdrl AS [S]ODN + VIN-treated cells. Results are from a representative experiment. In B. cells were untreated (Lane 1), or exposed to mdrl sense (Lane 2). SCR (Lane 3), or AS (Lane 4) [S]ODN (as described in the legend to Fig. 2). gp-170 levels were assessed with Western blot performed at the end of mdrl [S]ODN treatment using an anti-gp-170 monoclonal antibody. As a control of protein amounts loaded and blotted and of [S]ODN specificity for gp-170 protein down-regulation. HSP 72/73 and MYB protein levels were also evaluated on the same blot. Representative of three different experiments with similar results.

Fig. 3. Expression of mdrl mRNA (A) and gp-170 protein B in HL-60/Vinc cells treated with mdrl [S]ODNs. In A, cells were exposed to mdrl sense or AS [S]ODNs (as reported in the legend to Fig. 2). 0.5 μg/ml VINC (for 72 h), and mdrl sense or AS [S]ODNs followed by VINC. Control cells were left untreated. To assess the mdrl mRNA level, cells were harvested at the end of treatment, and total RNA was isolated and divided into two equal portions and separately amplified using RT-PCR (35 cycles) with mdrl and β-actin-specific primers. Lane 1. untreated cells; Lane 2. VIN-treated cells; Lane 3. mdrl sense [S]ODN-treated cells; Lane 4. mdrl AS [S]ODN-treated cells; Lane 5. mdrl sense [S]ODNs + VIN-treated cells; and Lane 6. mdrl AS [S]ODN + VIN-treated cells. Results are from a representative experiment. In B, cells were untreated (Lane 1), or exposed to mdrl sense (Lane 2). SCR (Lane 3), or AS (Lane 4) [S]ODN (as described in the legend to Fig. 2). gp-170 levels were assessed with Western blot performed at the end of mdrl [S]ODN treatment using an anti-gp-170 monoclonal antibody. As a control of protein amounts loaded and blotted and of [S]ODN specificity for gp-170 protein down-regulation. HSP 72/73 and MYB protein levels were also evaluated on the same blot. Representative of three different experiments with similar results.

The HL-60/Vinc human leukemia cell line selected in vitro for resistance to VINC exhibits cross-resistance to other MDR-related drugs commonly used as antineoplastic agents. Accordingly, we combined mdrl [S]ODNs with ADR and obtained reversal of ADR resistance (percentage of cell growth inhibition and IC50 value decrease) only when HL-60/Vinc cells were exposed to mdrl AS [S]ODNs followed by ADR (data not shown).

Fig. 2B shows the survival curves form methylcellulose colony assays performed on HL-60/Vinc cells treated with mdrl [S]ODNs and VINC (see "Materials and Methods"). The combinations sense + VINC and SCR + VINC did not cause any increase in the lethal effect of VINC, since the survival curves of cells treated with VINC alone, or sense + VINC, or SCR + VINC are superimposable and the surviving fractions have similar values (about 90% at 0.01 μg/ml and about 50% at 1 μg/ml). Only the treatment with the AS + VINC combination enhanced the lethal effect induced by VINC, as indicated by the decrease of the surviving fractions (60% at 0.01 μg/ml of VINC and only 7% at the highest VINC dose used). These results indicate that mdrl AS [S]ODNs are effective in sensitizing HL-60/Vinc-resistant cells to VINC, thus reversing MDR in vitro.

Expression of mdrl mRNA and gp-170 Protein Levels in mdrl [S]ODN-treated HL-60/Vinc Cells. In an attempt to correlate the antiproliferative effect of the various compounds with levels of mdrl mRNA, we evaluated mdrl mRNA expression in HL-60/Vinc cells immediately after treatment with mdrl [S]ODNs plus VINC (Fig. 3A). In comparison to untreated cells (Lane 1), cells treated with mdrl AS [S]ODNs (Lane 4) and mdrl AS [S]ODNs + VINC (Lane 6) exhibited a marked reduction of mdrl mRNA levels. No decrease in mdrl mRNA expression was detected after other treatments (Lanes 2, 3, and 5). The levels of β-actin mRNA were essentially unchanged in cells receiving different treatments, confirming the specificity of the effects induced by mdrl AS [S]ODNs. These experiments were performed only with the VINC dose of 0.5 μg/ml because the antiproliferative effect of the highest dose (1 μg/ml) was too marked when used in combination with the antisense [S]ODNs (Fig. 2A).

Because of the long half-life of mdrl protein and the small number of cells available after exposure to mdrl AS [S]ODNs + VINC, we assessed the ability of mdrl [S]ODNs to down-regulate mdrl protein levels only in [S]ODNs-treated cells.

As shown in Fig. 3B, there was no difference in the gp-170 protein levels in control, sense-treated, or SCR-treated cells (Lanes 1–3, respectively). However, a decrease in the gp-170 protein level was detected after exposure of HL-60/Vinc cells to mdrl AS [S]ODNs (Lane 4) at the same dose used to assess the effects on cell proliferation (Figs. 1 and 2). The levels of HSP 72/73 protein and MYB protein did not diminish in AS [S]ODN-treated cells, suggesting that the down-regulation of P-170 glycoprotein expression was a specific effect of the mdrl AS [S]ODNs treatment.

Effects of mdrl [S]ODNs and VINC on Survival of SCID Mice Given Injections of HL-60/Vinc Cells. To assess the ability of mdrl [S]ODNs given in combination with VINC to reverse MDR in vivo, SCID mice given injections of HL-60/Vinc leukemia cells were treated, by tail vein injection, with mdrl SCR or AS [S]ODNs for 10 days (1 mg/mouse/day) in combination with VINC for 3 days (20 μg/mouse/day) during [S]ODN administration. The control groups received SCR [S]ODNs, AS [S]ODNs, VINC, or no treatment. Fig. 4 reports the survival curves of HL-60/Vinc SCID mice treated with [S]ODNs and VINC individually or in combination. To better distinguish the different survival curves, the curves relative to the mice in the SCR-treated groups (A) were separated from those of the mice of the AS-treated groups (B). For a better comparison of the different treatments, the survival curves of the untreated and the VINC-treated mice are the same in A and B. The administration of VINC alone did not prolong the survival of HL-60/Vinc SCID mice, consistent with the in vitro resistance of the leukemia cells to the drug. The untreated mice were all dead 84 days after implantation of HL-60/Vinc cells,
whereas the mice in the VINC-treated group were all dead 91 days after the inoculation of leukemia. The median survival times were 56 and 70 days for the control and the VINC group, respectively; this difference was not statistically significant (see the legend to Fig. 4). As expected, the treatment with SCR [S]ODNs alone or SCR [S]ODNs + VINC did not affect the survival of SCID mice, both curves being very similar to those of the control mice (untreated and VINC-treated). The mice in the SCR-treated group were all dead within 85 days, and those in the SCR + VINC-treated group within 92 days from the inoculation of leukemia cells, with a median survival time of 71 and 77 days, respectively. The treatment with mdrl AS [S]ODNs was not able by itself to affect the survival of SCID mice (Fig. 4B). The mice were all dead 100 days after injection of leukemia cells, with a median survival time of 57 days (median survival time of untreated mice, 56 days). The statistical analysis of the control versus the AS curve gave a nonsignificant $P$ value of 0.45 (see the legend to Fig. 4), indicating that the observed delay in the death of the AS-treated mice compared with that of controls (about 20 days) was only apparent. These results confirm those obtained in vitro (Fig. 1), indicating that HL-60/Vinc cell growth was not affected by mdrl AS [S]ODNs by themselves both at low (Fig. 1A) and high doses (Fig. 1B). No significant differences were observed by comparing the survival curve of the AS-treated mice with that of mice treated with VINC alone (median survival time 57 and 70 days, respectively, and $P$ value not statistically significant). In contrast, the survival curve of the mice treated with the combination mdrl AS [S]ODNs + VINC is completely different from the others, with 60% of the animals in this group still alive 300 days after leukemia cell injection. The median survival time of the AS + VINC-treated mice was then 300 days, and the statistical analysis gave $P$ values all included in the significant and highly significant range with each of the comparisons: control versus AS + VINC, 0.005; AS versus AS + VINC, 0.0045; VINC versus AS + VINC, 0.03; AS + VINC versus SCR, 0.014; and AS + VINC versus SCR + VINC = 0.04.

Three hundred days after leukemia cell injection, surviving mice from the AS + VINC-treated group were sacrificed. Histopathological examination of hematopoietic and nonhematopoietic organs, as well as RT-PCR analysis of c-myb transcripts used as a marker of leukemia cell load, did not reveal the presence of tumor cells at a detectable level (data not shown). Thus, our in vivo results indicate that we have restored VINC sensitivity in HL-60/Vinc-resistant cells injected in SCID mice by treatment with mdrl AS [S]ODNs in combination with VINC.

Since the development of MDR by cancer cells still represents one of the major reasons for anticancer chemotherapy failure (1), the aim of the present study was to attempt modulation and eventually reversal of MDR in a human leukemia-resistant cell line, both in vitro and in vivo, using AS [S]ODNs targeted to the mdrl mRNA. We used HL-60/Vinc cells that were selected from HL-60 parental cells for resistance to VINC (13). These cells overexpressed the P-170-kDa mdrl gene product and exhibited cross-resistance to other antineoplastic drugs related to the MDR phenotype, most likely as a consequence of increased transport of anticancer agents out of cells and/or decreased intracellular accumulation (4). Different AS ODNs have been reported to selectively down-regulate p-170 glycoprotein expression (8–11), suggesting that their use might have a therapeutic potential in cancer management after the emergence of the drug-resistant phenotype. Since unmodified AS ODNs have a short half-life in biological systems (19, 20), in our in vitro and in vivo experiments, we used [S]ODNs which are relatively resistant to cleavage by nucleases (19, 20). Moreover, [S]ODNs have a good solubility in aqueous solution and hybridize efficiently with the target mRNA.

Using an 18-mer antisense [S]ODN targeting the region immediately downstream from the translation initiation codon, we observed a marked, but not complete down-regulation of gp-170 protein, consistent with its half-life of about 72 h (9, 10, 21). Nevertheless, the observed reduction in the gp-170 level was sufficient to block its drug efflux effect, as indicated by the complete restoration of the in vitro sensitivity to VINC (Fig. 2A and Table 1), upon treatment of HL-60/Vinc cells with mdrl AS [S]ODNs and VINC. On the other hand, the down-regulation of mdrl mRNA induced by the AS [S]ODNs was more evident and almost complete (Fig. 3A), suggesting that a 4-day continuous exposure to the AS [S]ODNs was sufficient to obtain a strong inhibition of its expression, even if the mdrl mRNA is abundant and has a relatively long half-life of 4–5 h (22).

The observed in vitro reversal of MDR correlated well with the
results obtained in vivo using SCID mice given injections of the drug-resistant leukemia cells. In vivo, mdrl AS S]ODNs given in combination with VINC was able to sensitize the resistant leukemia cells to the effect of VINC and thus to reverse MDR and prolong survival of mice with leukemia. The prolonged survival of mice with leukemia by means of sequence-specific S]ODNs in combination with VINC supports a specific mechanism of action of mdrl AS S]ODNs in vivo. However, S]ODNs also exhibit sequence-independent effects that may reflect the ability of such compounds to interact with cellular and extracellular proteins (23, 24). Thus, we cannot exclude the possibility that an apparently sequence-specific effect is indeed sequence independent or that sequence-independent and sequence-specific effects coexist. In conclusion, our studies raise the possibility that S]ODNs (or newly developed analogues) targeted to the mdrl mRNA might be useful in the attempt to reverse MDR in cancer patients.

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References

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