Functional comparison of single- and double-stranded *mdr1* antisense oligodeoxynucleotides in human ovarian cancer cell lines.

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Received August 17, 2005; Revised September 21 2005; Accepted September 22, 2005; Published September 23, 2005

ABSTRACT

PURPOSE. P-glycoprotein mediated multidrug resistance presents a major obstacle in the successful therapeutic treatment of solid tumors such as ovarian cancer. Among the more promising techniques used to overcome multidrug resistance in ovarian cancer, is the transcriptional suppression of P-glycoprotein by antisense oligodeoxynucleotides (ODNs). To design more potent antisense ODNs, we explored the concept that double-stranded antisense ODNs may offer advantages in stability and potency over single-stranded in analogy to double-stranded siRNA. **METHOD.** Single-stranded phosphorothioate antisense ODNs against the human *mdr1* gene were compared to the duplex of the active antisense and sense sequence of the same length. Concentration dependant effects on P-glycoprotein (Pgp) expression and functionality were quantitatively compared in the Pgp overexpressing ovarian cancer cell line A2780/Adr and its parental cell line A2780. Antisense ODNs were ^{111}Indium- and fluorescein isothiocyanate-conjugated for stability, cellular uptake and nuclear localization studies. Duplex formation significantly enhanced transcriptional inhibition of Pgp surface expression and functionality. Cellular uptake and distribution to the nucleus was improved when utilized as double-stranded DNA. **CONCLUSION.** Novel findings from this study suggest that double-stranded antisense ODNs more effectively inhibit target protein expression and consequently enhance chemoresponsiveness through improvements in cellular uptake and distribution to the nucleus.

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INTRODUCTION

Antisense oligodeoxynucleotides (ODNs) are powerful tools for the selective, sequence-specific regulation of gene expression (1). Though extensively investigated and convincing in theory, practical applications have proven to be rather challenging (2). To date, only one antisense-based drug has been released to the market (3). Whereas some successes have been described for target genes that are present at relatively low levels (4, 5), the application of antisense technology for target genes with high expression has proven to be difficult due to excessive toxicity, insufficient *in vivo* stability and cellular uptake (4, 6). Since their introduction, numerous chemical modifications of ODNs have been described in efforts to increase their stability and specificity. In the first generation of ODNs, a single oxygen atom at each phospate group was replaced with a sulfur atom producing phosphorothioate (PS) ODNs, rendering them relatively nuclease resistant. More efforts have been placed to develop second and third generation antisense ODNs including the 2'-O-alkyl RNA derivatives (7) and particularly the phosphorodiamidated morpholino oligomers (8). While those newer antisense technologies offer advantages such as reduced non-specific effects and more efficient target protein arrest, the conventional PS ODNs are still the best known and most widely used in research and therapy (6, 9-11).

Interest in the antisense field has recently exploded subsequent to the discovery that short double-stranded siRNA molecules could be used to specifically suppress gene expression within cells. The double-stranded siRNA molecules form 3'-overhangs that specifically inhibit gene expression. Recently, functional effects of single-stranded (ss) and double-stranded (ds) siRNA have been compared and it has

Abbreviations:
ODNs, Oligodeoxynucleotides
PS, Phosphorothioate
ss, single-stranded
ds, double-stranded
mdr1 , multidrug resistance gene
Pgp , P-glycoprotein
A2780/Adr, Adriamycin resistant A2780
A, antisense
S, sense
R, random
PBS, phosphate-buffered saline
FACS, fluorescence assorted cell sorting
DTPA, diethylenetriaminepentaacetic acid
FITC, fluorescein isothiocyanate
DAPI, 4,6-diamidino-2-phenylindole
DNR, Daunorubicin

been demonstrated that double -stranded siRNA possess unexpectedly high efficacy and stability both in cell culture (12) and in animal models (13). While much research has been directed toward examining these double-stranded RNA molecules, little effort has been placed toward examining potential benefits in the stability and activity of double-stranded DNA based antisense ODNs. Recently, an enhanced cellular uptake of a multidrug resistance gene (mdr1) ODN duplex consisting of a phosphorothioate antisense sequence combined with a shorter non-phosphorothioate sense sequence was reported, suggesting that double-stranded ODN delivery may provide an effective means for increasing cellular uptake of antisense oligonucleotides (14).

Antisense ODNs directed against the *mdr1* gene have been well described and are reported to effectively inhibit expression of *mdr1* and the protein it encodes for, P-glycoprotein (Pgp) (15, 16). Overexpression of Pgp, a member of the ATP-binding cassette transporter family, is a common cause of multidrug resistance in many types of tumors including ovarian and breast cancer. This membrane bound efflux pump expels chemotherapeutic agents from cells, resulting in decreased intracellular drug concentrations and loss of efficacy (17-19). ODNs, designed mostly against the region of the initiation codon of *mdr1*, have been demonstrated to inhibit the Pgp mediated multidrug resistant phenotype *in vitro* in cell culture (10) and *in vivo* in human tumour xenografts (20). Due to relatively high Pgp levels present in multidrug resistant tumor cells advances in the efficacy, stability and cellular uptake of antisense ODNs are needed to successfully apply this technique for therapeutic use.

Using a human *mdr1* antisense sequence that has previously been used by others to inhibit Pgp expression (10, 21); we investigated the potential advantages in stability, cellular uptake, toxicity and efficacy of double-stranded versus single-stranded ODNs. Phosphorothioate antisense and sense ODN of the same length were utilized and compared in the resistant A2780/Adr human ovarian cancer cell line which highly expresses Pgp, as well as its sensitive parent A2780 cell line. Novel findings from this study are the first to demonstrate that efficacy of *mdr1* ODNs is increased when used as double-stranded phosphorothioate ODNs versus single-stranded. Enhanced suppression of Pgp expression was confirmed by functional analysis. Based on fluorescence microscopy analysis of tagged ODNs, increased cellular retention and nuclear localization of the ds ODN is the likely mechanism involved.

MATERIALS AND METHODS

Cell culture. Adriamycin resistant (A2780/Adr) and parent A2780 ovarian cancer cell lines were purchased from ECACC, UK. Cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 μg/ml penicillin and streptomycin and incubated in a 5% CO_2 atmosphere at 37°C. A2780/Adr were incubated with 10μM Adriamycin every 10 passages.

Pgp overexpression in A2780/Adr cells was confirmed by RT-PCR (Fig. 1) using previously described methods (22). Briefly, total RNA was isolated from A2780/Adr and A2780 cells using the TRIzol extraction kit (GIBCO-BRL; Life Technologies, Gaithersburg, MD) according to manufacturer's protocol. cDNA was synthesized from 0.5 μg of RNA using the First Strand cDNA synthesis kit (MBI Fermentas, Flamborough, ON, Canada). *Mdr1* mRNA levels were determined by semiquantitative RT-PCR using the following primer sequences: forward primer: 5'-GTA CCC ATC ATT GCA ATA GC; reverse primer 5'-CAA ACT TCT GCT CCT GAG TC and normalized to GAPDH (forward primer: 5'-ACC ACA GTC CAT GCC ATC AC-3; reverse primer: 5'-TCC CAC CAC CCT GTT GCT GTA-3. RT-PCR products were separated on 2% agarose gels by electrophoresis, stained with a 1:10000 SYBR gold and visualized using Kodak Digital Science Image Analysis Software.

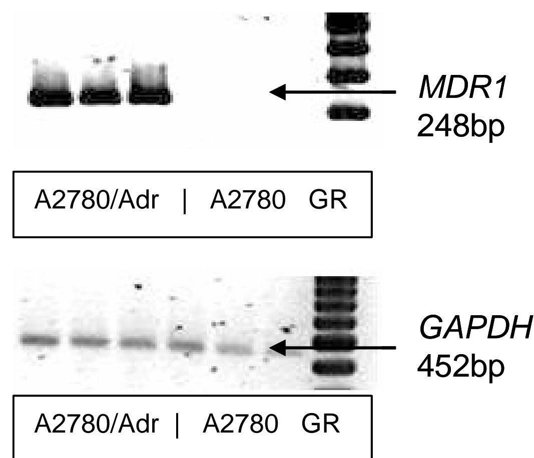

Figure 1. RT-PCR analysis of *mdr1* expression. A representative RT-PCR gel depicting relative *mdr1* gene expression in A2780/Adr and A2780. RT-PCR was performed and PCR products were separated and visualized as reported in Method. GAPDH was used as housekeeping gene. Lanes 1-3 contain A2780/Adr, lanes 4-6 A2780 samples and lane 7 is the DNA ladder (100 bp Gene Ruler-GR).

Product sizes were verified by a 100 bp gene ruler (Invitrogen, Carlsbad, CA). Linear conditions were established from standard curves as previously described (23).

Transfection with oligodeoxynucleotides. Phosphorothioate ODNs with aminohexyl modifications at the 5'-end were purchased from Invitrogen, (Carlsbad, CA). Sequences used were; Antisense (A): Aminohexyl-5'-CCA TCC CGA CCT CGC GCT CC-3', Sense (S): 3'-GGT AGG GCT GGA GCG CGA GG- 5'-Aminohexyl and Random (R): Aminohexyl -5'-GCT CCC CCA CGC GCC TCC AT-3'. Double-stranded AS-S duplexes were formed by combining 100 μM stock-solutions of ss AS and S and incubating at room temperature for 10 min at concentrations between 25-250 nM. Formation of AS-S duplexes was routinely verified on 4% polyacrylamide gels. A2780 cells were plated onto 6 well plates at a concentration of 33,000 cells per well and incubated for 24 hour prior to treatments with increasing concentrations of AS and double-stranded AS-S ODNs; single-stranded random ODNs were used as controls. SuperFect (Qiagen, Mississauga, ON) was added (ratio 12µl per 1µg DNA) according to manufacturer's instructions. Cells were incubated with the tranfection mixture for 6 hours and solution was replaced with fresh media. Cells were treated daily for three consecutive days (72 h).

The stability of double-stranded AS-S and single-stranded AS *mdr1* ODNs was examined by incubation in PBS (PBS) containing 5% fetal bovine serum (Sigma, Kanata, ON) at 37°C. Samples were removed at various time intervals (3-24h) and enzyme reactions terminated by heating at 95°C for 10 min (24). All samples were subsequently loaded onto a 4% polyacrylamide gel, run at 150 V and visualized using the SYBR gold nucleic acid stain. Freshly prepared ODNs served as controls.

Effect of ODN on Cell Viability and Proliferation. Cell number as a measure of cell viability was assessed at 72 hours after final ODN treatments. Plated cells were trypsinized, centrifuged at 1000 g, 4°C for 4 min, redissolved in medium and aliquots analyzed on a Casy 1 electronic particle counter (Schaerfe Systems GmBH, Reutlingen, Germany). Only intact cells (size between 10 and 40 µm) were counted. To ensure evaluation of overall ODN-related toxicities, untreated A2780 cells were used as controls. Changes are reported as percentage of untreated controls.

Cell proliferation was further assessed using the MTT assay. This assay measures mitochondrial enzyme succinyldehydrogenase activity in viable cells. Cells from each treatment group were plated onto 96 multiwell plates (100,000 cells/well) for 3 hours (5% CO_2, 37°C), 20 µl of MTT solution (5 mg/ml) added and incubated for 1 hr. Cells were then solubilized with isopropanol-HCl (1:300) and absorption of solubilized formazan was measured using a BMG Fluostar (BMG LABTECH GmbH, Offenburg, Germany). Formazan absorbance was calculated by measuring absorbance maximum at 595nm and subtraction of background absorbance at 690nm. Cell proliferation was calculated as a percentage of controls.

P-Glycoprotein surface expression. After final ODN treatments, cells were washed several times with PBS and attached cells were then trypsinized, counted and prepared for antibody staining. 10^6 cells were washed with 1 ml of buffer (PBS w/ 0.5% BSA) and dissolved in 1 ml of staining buffer (PBS, 0.5% BSA, 0.1% NaN_3) containing 20 µl FITC labeled monoclonal anti human Pgp antibody (BD Biosciences, USA). Tubes were protected from light and incubated on ice for 40 min, cells were then washed with 1 ml of ice-cold staining buffer to remove unbound antibody and resuspended in buffer. Binding of FITC-labeled Pgp-antibody was analysed on a Becton flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) with excitation at 488 nm and emission collected through a 530/15 band pass filter for FITC (FL1-H). 10000 events were collected while gating on physical parameters to exclude cell debris. The number of events within the gate for intact cells remained consistently well above 75% for all ODN treatments of 25-200 nM and were consistent with cell viability studies. A lower cell intact fraction of 53% was seen only in the 250 nM ds AS-S treated cells. Data was quantified using the Cell Quest Pro and Win MDI software. Autofluorescence of A2780/Adr and A2780 cells were tested to be identical. As both cell lines do not express BCRP, non-specific binding to the BCRP binding antibody BXP-21 (Abcam, Cambridge, MA) and anti-mouse Ig fluorescein-linked whole antibody (Amersham Biosciences, Piscataway, NJ) was used to evaluate unspecific binding. Minimal non-specific binding was detected and differences in fluorescent intensities were not seen between the two cell lines (data not shown).

Daunorubicin chemosensitivity. Cells treated for 72 hr with 50 nM or 100 nM ODNs and untreated controls were plated in media at 50,000 cells/well into 96 multiwell plates and incubated with increasing concentrations of Daunorubicin (100 nM - 1mM in 10

µl) for 72h under standard conditions (5% CO_2 at 37°C). Cell proliferation and viability was then assessed using the MTT-assay. Concentrations of Daunorubicin required to decrease cell proliferation by 50% (IC_{50} values) were determined for each ODN treatment group from the concentration-response curves of the MTT assay. The concentration-response curves and IC_{50} values were obtained by nonlinear regression assuming a sigmoidal dose response curve with variable hill slope, generated with GraphPad Prism 3.0 Software.

Intracellular Daunorubicin uptake. Using previously described methods (25, 26); cellular uptake of the fluorescent drug Daunorubicin was analyzed by FACS. Briefly, 10^6 ODN treated cells were immediately diluted in 2 ml phenol red-free RPMI medium and incubated for 1h at 37°C. Daunorubicin (10µM) was added and cellular daunorubicin uptake was measured every 30 min for 3 h on a Becton flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) with excitation at 488 nm and emission collected through a 585/22 band pass filter for yellow daunorubicin (FL2-H). 5000 event were collected while gating on physical parameters to exclude cell debris and sample analysis was repeated three times. Data was analyzed by Cell Quest Pro software and accumulation plateaus were generated using GraphPad Prism 3.0 software.

Cellular uptake of radiolabeled ODNs. Antisense ODNs containing an aminohexyl group at the 5'-end were combined with a 100-fold molar excess of DTPA anhydrate (Sigma, Kanata, ON) at room temperature for 30 min as previously described (27). DTPA-derivatized ODNs were purified using size-exclusion chromatography on a P-2 mini-column (Bio-Rad, Mississauga, ON) and eluted with 150 mM of sodium chloride. Purified DTPA-derivatized ODNs were labeled with ^{111}Indium acetate (MDS Nordion Inc. Vancouver, BC) by incubation for 30 min at room temperature. A specific activity of approximately 0.25 MBq/µg was obtained. A radiochemical purity of >95% was determined by silica gel instant thin-layer chromatography (ITLC-SG, Gelman, Ann Arbor, MI) using 100 mm sodium citrate pH 5.0. For uptake experiments, A2780/Adr and A2780 cells were seeded on 96 well plates at a density of 100,000 cells/well and incubated for 24h at 37°C and 5% CO_2. Cells were treated with increasing concentrations (25-250 nM) of [^{111}In]-DTPA-labeled single-stranded AS or double-stranded AS-S *mdr1* ODNs for 6h. Media was subsequently collected and cells were washed with ice-cold buffer and trypsinized. Radioactivity of media and cell lysates were counted using a gamma counter (Packard Cobra II®, Series Auto-Gamma ®, GMI Inc., Minnesota, USA). The amount of cellular uptake was expressed in cpm of cellular bound ^{111}Indium. The stability of [^{111}In]-DTPA-labeled AS ODNs was verified by separating 5 µl of cell lysates on silica gel instant thin layer chromatography.

Intracellular distribution of fluorescent-labeled ODNs. Intracellular distribution of fluorescent-labeled ODNs was visualized in attached viable cells using fluorescence microscopy. For FITC labeling of AS ODNs, a 1mg/mL FITC-isothiocyanate solution was prepared in 100mM sodium bicarbonate buffer (pH 9). Aminohexyl-modified AS ODNs were incubated with 300µM of FITC-isothiocyanate-solution (1:100 ratio) for 30 min at room temperature in the dark. FITC-labeled ODNs were purified by size-exclusion chromatography on a P-2 column. A2780 and A2780/Adr cells were grown on glass supports. At 60% confluency, cells were treated with 200nM of double-stranded AS-S and single-stranded AS FITC-labeled *mdr1* ODNs for 3 hr. For nuclear morphology analysis, cells were incubated with a 1 µg/ml DAPI in PBS for 5 min. Cells were washed and examined under a 100x 1.30 oil immersion objective using a Nikon Eclipse E400 microscope (Nikon, Mississauga, ON).

Data analysis. Statistical analysis was performed using the unpaired Student's *t*-Test (Excel; Microsoft, Redmond, WA) and analysis of variance (GraphPad Prism 3.0, GraphPad Software, San Diego, CA). Data are presented as the mean ± standard deviation (p-levels: * < 0.05; ** <0.01). IC_{50} values were generated by nonlinear regression assuming a sigmoidal dose response curve with variable hill slope, generated with GraphPad Prism 3.0 Software.

RESULTS AND DISCUSSION

Inhibition of cell surface expression of P-glycoprotein. The effects of double-stranded AS-S and single-stranded AS *mdr1* ODNs on Pgp expression were compared using flow cytometry. A conventional single-stranded random control ODN was used to evaluate nonspecific effects. Preliminary studies examined dose and time-dependency of Pgp expression with the ODN treatments. Taking toxicity into account, we found that significant reductions in Pgp surface expression were seen after 48 h with the most pronounced suppression seen at 72 h (data not shown). As the half life of Pgp is 14 h, this corresponds to

treatment periods of 3-5 Pgp half lives (28). We observed significant, dose-dependent reductions in Pgp surface expression in cells treated with single-stranded AS and double-stranded AS-S preparations (Fig. 2). The double-stranded AS-S ODN was significantly more effective than single-stranded AS at all tested concentrations with the largest difference in suppression seen at the lowest concentration (23 % increase in Pgp suppression at 25nM). On the other hand, the strongest suppression of Pgp surface levels was seen in cells treated with 250 nM of double-stranded AS-S. Representative FACS histograms, demonstrating reduced Pgp surface expression in treated cells, are depicted in Figure 3. Histograms indicate homogeneity of the ODN treated cell population, since the peaks tail slightly but do not divide into a second population.

Daunorubicin chemo sensitivity and intracellular accumulation. Changes in Pgp surface expression were confirmed by functional studies examining chemosensitivity and intracellular accumulation of the Pgp substrate daunorubicin (DNR). DNR accumulation and chemosensitivity have been shown to directly correlate with Pgp expression (29, 30). Chemosensitivity was determined after treatment regimens of 50 nM or 100 nM using the MTT assay and are reported as IC_{50} values in Table 1. As compared to single-stranded AS, IC_{50} values for double-stranded AS-S were significantly decreased. This indicates a higher chemosensitivity and lower resistance in cells treated with double-stranded AS-S ODN regimens.

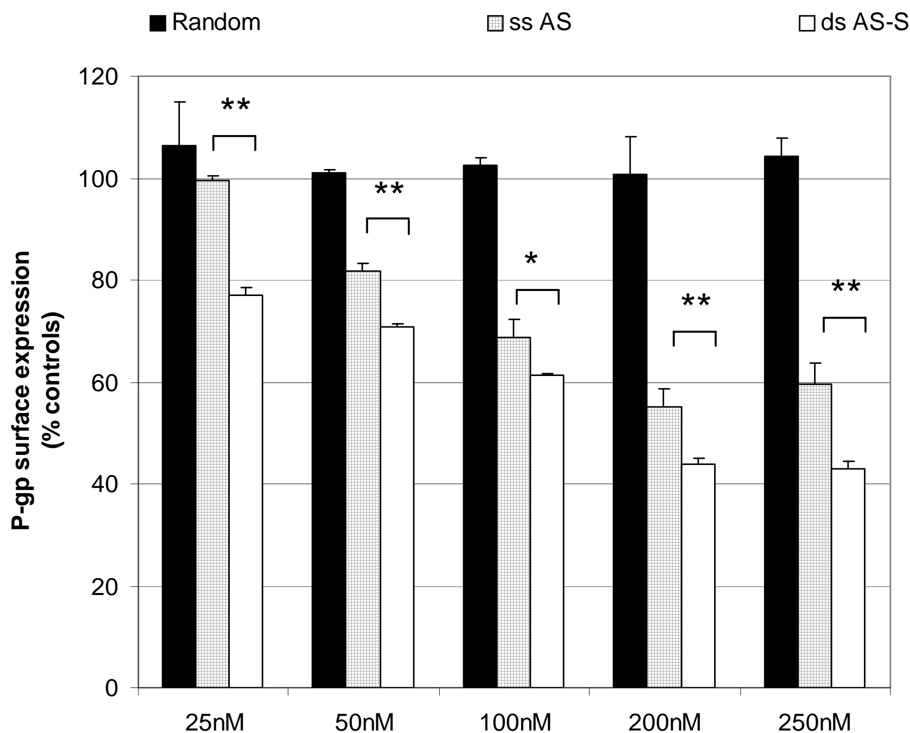

Figure 2. Effect of single-stranded antisense (ss AS) and double-stranded (ds AS-S) *mdr1* ODNs on P-glycoprotein surface expression. A2780/Adr cells were transfected with different concentrations of ODNs as described in methods. A conventional single-stranded random sequence was included to evaluate non-specific effects on protein expression. For detection, a FITC-labeled monoclonal human P-glycoprotein antibody was used with flow cytometry detection. Cells were gated for the intact population and the geometric mean of 10000 events was recorded. Results are expressed as a percentage of untreated A2780/Adr and represent the average of three individual experiments.

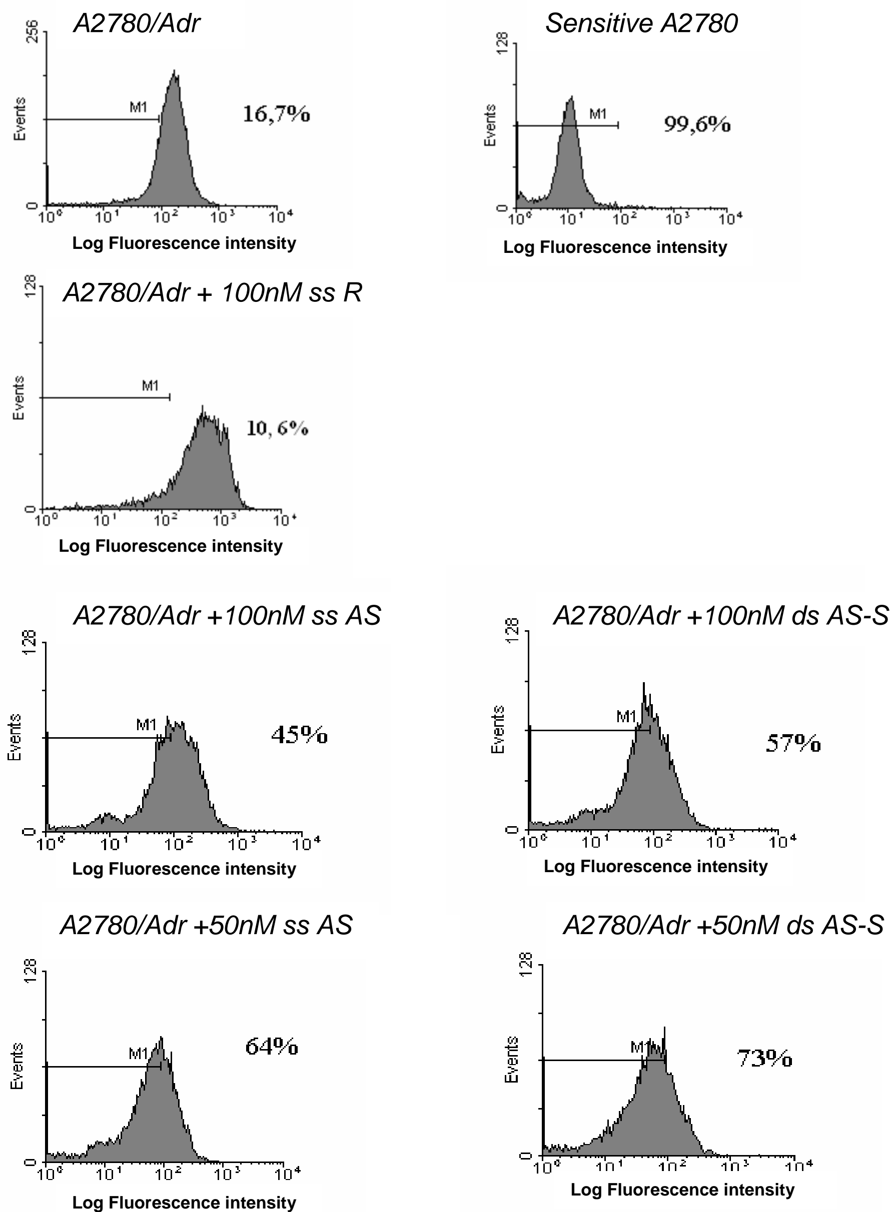

Figure 3. Representative histograms of Pgp surface expression analysis. The effects of treatments with 50 and 100 nM double-stranded (ds) and single-stranded (ss) AS *mdr1* ODNs on Pgp surface expression in A2780 are presented. Random control, untreated resistant A2780/Adr and sensitive A2780 were used as negative and positive controls. Cells were prepared by incubation with a FITC-labeled monoclonal anti-P-glycoprotein-antibody and Pgp expression was detected by flow cytometry analysis. Acquisition was set to 10000 events and results depict the viable/ intact cell population as routinely gated in the forward and side scatter plot. The fluorescent intensity of the bound FITC antibody was detected in the FL1-H channel. In the histograms, the gate M1 is placed to include sensitive A2780 cells (99.6%) and excluding Pgp expressing A2780/Adr cells (16.7%). Percentages in each panel represent the proportions of cells (=events) that do not express Pgp (A2780).

The increased chemosensitivity of DNR in ODN treated cells was associated with enhanced intracellular accumulation of DNR as detected by FACS analysis (Fig. 4). Differences in Pgp expression were clearly illustrated by the 3 fold differences in DNR accumulation between A2780/Adr and A2780 cells (Fig. 4A). Whereas ODN treatments did not significantly alter DNR

accumulation in A2780 cells, DNR levels were appreciably increased in A2780/Adr cells. In A2780/Adr cells, we detected significantly higher accumulation of DNR in cells treated with double-stranded AS-S ODN (Fig. 4B). Whereas treatment of cells with 50 nM of the AS-S duplex resulted in a 30% increase in DNR accumulation, single-stranded AS did not significantly impact DNR levels. DNR accumulation was further increased by more than 2 fold in cells treated with 100nM of double-stranded AS-S ODN. By comparison, DNR accumulation was increased to a maximum of 145% in cells treated with 50- 250 nM of the single-stranded AS preparation.

Table 1. Daunorubicin chemosensitivity. Daunorubicin chemosensitivity was determined in A2780/Adr cells treated for 72 hr with single-stranded AS, double-stranded AS-S *mdr1* ODNs or random controls. Cells were incubated with Daunorubicin or PBS and cell vitality was determined with the MTT-assay. The concentration that produces 50% inhibition of cell vitality (IC_{50}) was calculated. Results were generated from means of six data points. two separate experiments. * indicates a $p < 0.05$ between ss AS and ds AS-S ODN treatment.

Treatment	$IC_{50} \pm$ SEM / µM		
	ss AS	**ds AS**	**PBS**
Control			37.9 ± 0.8
50 nM	19.1 ± 0.8	8.3 ± 0.8 *	
100 nM	9.7 ± 0.9	2.8± 0.8 *	

Cellular toxicity of ODN transfection. Cellular toxicity has been associated with the exposure to PS ODNs (4) and remains a major challenge in antisense application. We examined whether double- and single-stranded preparations of PS ODNs differ in toxicity. Cell viability status and cell number counts as analyzed on a Casy 1® cell counter, did not detect any significant differences between the single- and double-stranded ODN preparations (Fig. 5A). Decreased cell viability was seen in cells treated with 250 nM of ss-AS or ds-AS-S ODN that was consistent with cell gating in the FACS studies. On the other hand, cell proliferation was significantly lower in cells treated with single-stranded AS as compared to the double-stranded AS-S preparations (Fig. 5B).

Intracellular ODN stability, accumulation and localization.

To investigate the cellular mechanism underlying the enhanced effectiveness of ODNs to suppress Pgp expression and function when presented as a double-stranded duplex as compared to a single-strand, we examined the intracellular accumulation, cellular localization and stability of these preparations. ODN stability, as routinely examined in bovine serum albumin (25), did not detect differences between the stability of the single- or double-stranded AS ODN preparations over a 24 h time period. All samples remained stable under these conditions and no degradation products were detected. As fresh ODN are replaced in the media every 24h during our treatment periods, this implies that differences in AS activity are not due to changes in stability.

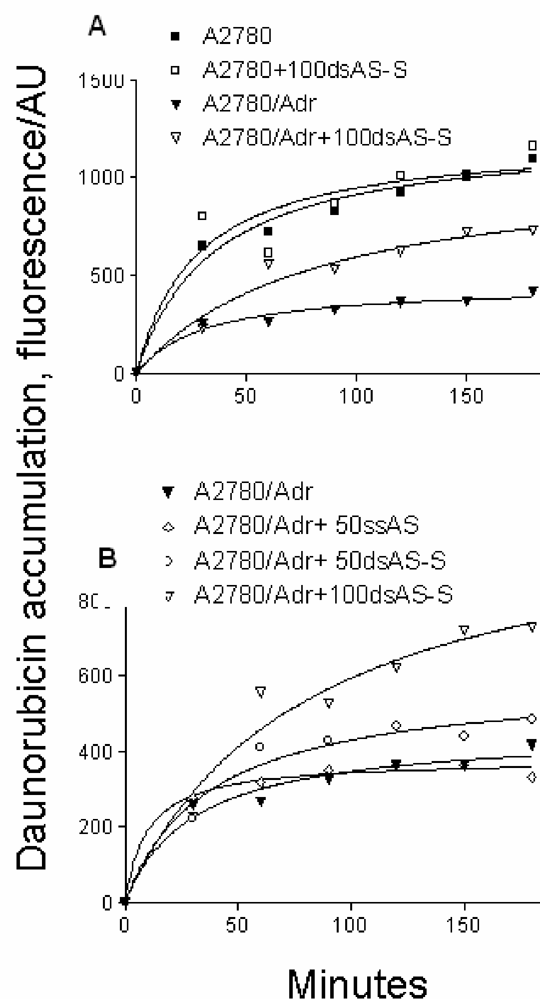

Figure 4. Effect of Antisense Treatments on Daunorubicin accumulation. Daunorubicin accumulation is shown in **A.** resistant A2780/Adr and A2780 cells transfected with 100 nM of double-stranded AS-S *mdr1* ODNs. **B.** in A2780/Adr cells transfected with 50 and 100 nM of double-stranded and single-stranded AS *mdr1* ODNs. Cells were incubated with 10 µM of Daunorubicin or PBS in a single cell suspension. In order to evaluate ds AS-S ODN effects on non-Pgp expressing cells, A2780 cells incubated with 100nM of ds AS-S ODNs were used as controls. After different time points an aliquot was removed and flow cytometry analysis was performed. Acquisition was set to 5000 events and gated for viable/ intact cell population. Data represent the geometric mean and similar results were obtained in two separate experiments.

To determine possible differences in cellular uptake of the AS preparations, AS ODNs were radiolabeled with ^{111}Indium. A2780/Adr and A2780 cells were incubated with different concentrations of [^{111}In]-DTPA-labeled AS or AS-S ODNs. As compared to the single-stranded AS, we observed a significant increase in the intracellular accumulation of ^{111}Indium in cells treated with the double-stranded AS-S ODN (Fig. 6). This was significant at all concentrations above 100nM and detectable in both A2780/Adr (Fig. 6A) and A2780 (Fig. 6B) cells. While a substantially higher accumulation of the radiolabeled double-stranded AS-S duplex was seen in A2780/Adr cells as compared to the A2780 cells, cell line associated differences in the accumulation of the single-stranded AS were not seen. Of note, we observed a higher variability in cellular uptake when using 250 nM of ODN. This may be due to either saturation of active transport mechanisms or toxic effects. Identification and stability of [^{111}In]-DTPA-labeled ODNs were confirmed by thin layer chromatography which verified detection of ODN-associated rather than free ^{111}Indium. Thin layer chromatography analysis of cell lysates demonstrated that more than 75% of radioactivity was associated with the ODN bound ^{111}Indium for both preparations.

Cellular uptake and distribution were further examined by fluorescence microscopy. As shown in Figure 7, the cellular uptake and retention of FITC-labeled AS was dramatically greater in A2780/Adr, as compared to the non-Pgp expressing A2780 cells (Fig. 7C, D). Increased fluorescence was observed in cells treated with the double-stranded AS-S preparation (Fig. 7A) compared to the single-stranded AS preparation (Fig. 7B). The nuclear localization of FITC-labeled ODNs was confirmed through overlapping DAPI (Fig. 8B) and FITC (Fig. 8C) stains. Likewise, a stronger fluorescent signal was detected in the nuclei of cells treated with the double-stranded AS-S preparation (Fig. 8C) as compared to cells treated with the single-stranded AS (Fig. 8D).

CONCLUSIONS

In this study, we report an increased activity of double-stranded versus single-stranded *mdr1* AS ODNs. Suppression of protein levels and functionality was increased along with chemoresponsiveness in the Pgp overexpressing A2780/Adr human ovarian cancer cell line. Effects of the double-stranded *mdr1* AS were dose-dependant and directly associated with changes in Pgp functionality, as measured by both increased accumulation and chemosensitivity towards the Pgp substrate, daunorubicin. While double-stranded RNA molecules have been extensively studied and successfully applied in the past, double-stranded DNA have received very little attention. Only recently, an increased activity of a double-stranded DNA preparation, consisting of a duplex between an active antisense strand and an easily degradable shorter complementary sense strand, was reported. This study demonstrated higher activity towards the suppression of cell surface protein but did not examine further effects on protein functionality and efficacy (14). Using fully active, phosphorothioate

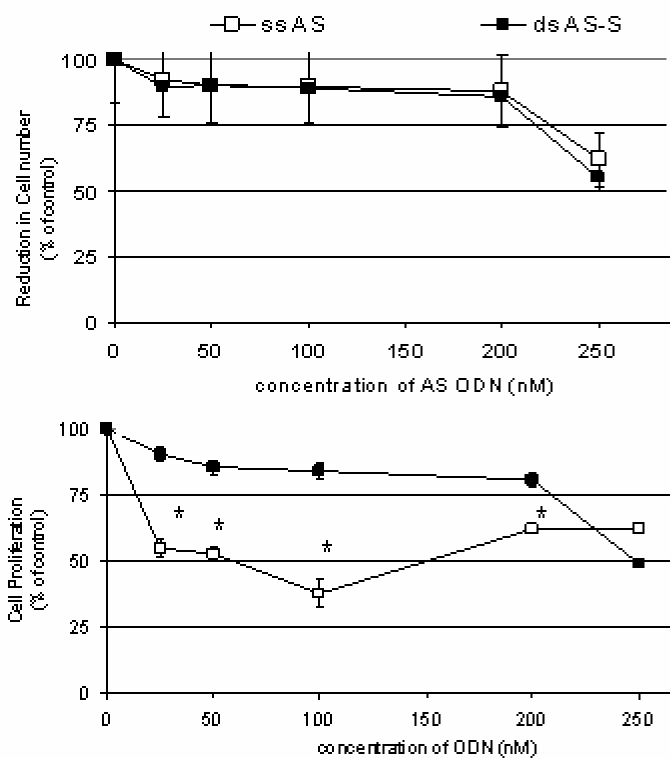

Figure 5. Effect of Antisense Treatments on (A) Cell Viability and (B) Cell Proliferation. Results depict effects of transfection (72h) with different concentrations of single-stranded AS and double-stranded AS-S *mdr1* ODN on A2780/Adr cells. To evaluate direct ODN related toxicity, untreated A2780/Adr cells were used as controls. Viability was determined by cell counts in Casy1® and are expressed as the reduction in cell number as a % of untreated controls. Cell proliferation was determined using the MTT assay. Data is expressed as the mean ± SD of three data points. Similar results were obtained in three individual experiments. No significant differences were detected.

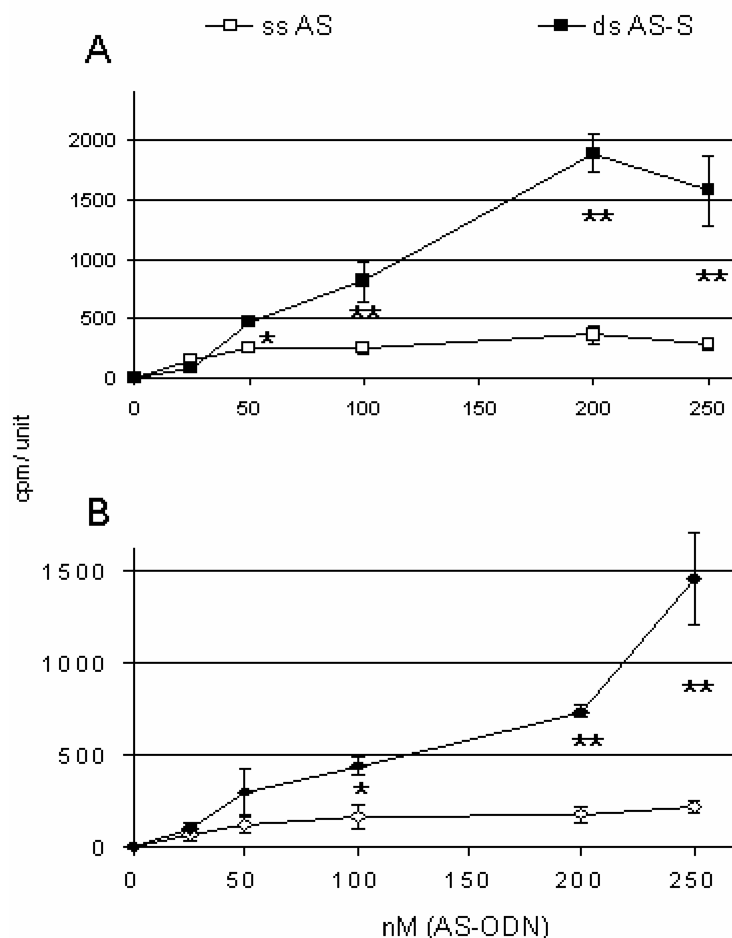

Figure 6. Intracellular uptake of ^{111}In-DTPA-labeled ODNs. Cellular bound recovery of ^{111}In-DTPA-labeled AS in the presence and absence of equimolar amounts of unlabeled sense ODNs in **A.** resistant A2780/Adr and **B.** sensitive A2780 cells is presented. Cells were transfected with different concentrations of single-stranded and double-stranded *mdr1* ODNs for 6 h. Results are expressed as amounts of intracellular bound ^{111}Indium in cpm per 126.6mm^2 confluent cells (area of 1 well = 1 unit) and represent the average of three individual experiments.

AS and S ODNs in single- and double-stranded preparations we were able to directly compare treatment efficiencies for the preparations. Interestingly, double-stranded ODNs were more effective but did not increase toxicity.

The increased activity of double-stranded ODNs was likely due to an increase in cellular accumulation and localization to the nucleus. This was confirmed in studies using either ^{111}In-DTPA-labeled or FITC-labeled ODNs that demonstrated increased intracellular levels. The results indicate that double-stranded ODN preparations strongly increase the delivery into cells and most likely into the nucleus, as visualized by fluorescence microscopy. Cellular uptake of DNA is generally thought to occur through receptor-mediated endocytosis. The fact that we observed increased nuclear and cellular uptake of the double-stranded DNA as compared to single-stranded DNA suggests that there may be a difference in the affinity to active transporters or additional uptake processes for double-stranded DNA. This seems plausible, as many receptor-mediated endocytosis pathways exist (DNA receptor protein, nucleic acid binding receptor-1, heparin binding protein, nucleoli-like

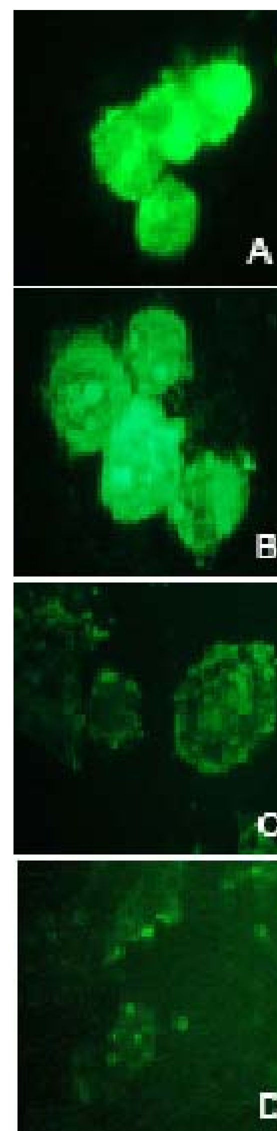

Figure 7. FITC-Fluorescent microscopy analysis of intracellular ODN in A2780/Adr (A, B) and A2780 (C, D) cells. Cells were transfected with double-stranded AS-S *mdr1*ODNs (A, C) and single-stranded AS *mdr1* ODNs (B, D) for 3 h.

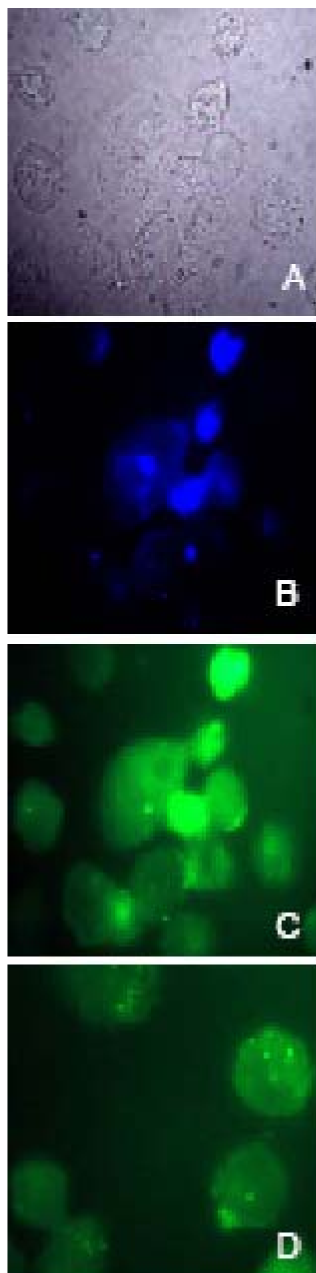

Figure 8. Fluorescent microscopy of nuclear ODN delivery. Nuclear localization analysis of oligonucleotides. Phase contrast (A, grey); DAPI nuclear stain (B, blue) and FITC-fluorescence (C, green) microscopy images were taken of A2780/Adr cells. Overlapping stains in B and C indicate nuclear localization of FITC-labeled ODNs. Furthermore, florescent images of cells transfected with double-stranded (C) FITC-labeled AS-S and single-stranded (D) AS *mdr1* ODNs are compared.

proteins, and porin-like proteins) (31). Chemically, the single-stranded DNA molecule is a highly polar electrolyte with a high negative charge density and a s urrounding cloud of counter ions in its vicinity (32). In the duplex conformation, even though the charge density is increased, the double-strand can more effectively neutralize this charge than the single-strand. Due to this more effective charge neutralization, the transport of the duplex through the non-polar environment of the membrane is facilitated and therefore increased in comparison to the transport of the single-strand. Furthermore, conformational changes of the AS sequence when presented as double-stranded DNA might potentially increase binding to target RNA and activation of RNase H, such as that reported for double-stranded siRNA (33). On the other hand, it is possible that both antisense and sense strands, once delivered to the nucleus could each interact with nuclear targets that alter gene expression. These hypotheses however, need to be confirmed with further experimentation.

It could also be argued that alterations in intracellular stability could be responsible for the enhanced cellular retention of the double-stranded ODNs. However, no differences in stability could be detected when analyzed using radiolabeled ODNs with thin layer chromatography analysis or via enzymatic incubation with gel electrophoretic separation and detection.

From these findings, we conclude that the formulation of double-stranded complementary phosphorothioate ODNs may have significant advantages over the use of single-stranded AS ODNs. Through advantages in cellular uptake and localization and potential contribution of the sense strand, double-stranded *mdr1* ODNs may be more effective in the suppression of Pgp without increasing toxicity. Ultimately, this could increase chemoresponsiveness to antineoplastic agents and hence provide improvements in overcoming Pgp-mediated multidrug resistance. Whether this approach holds true for differently modified oligonucleotides and *in vivo* applications is a question that will be of further interest in the antisense field. For future antisense applications of phosphorothioate oligonucleotides in the multidrug-resistance field, double-stranded ODN delivery may be a suitable approach.

ACKNOWLEDGMENTS AND SOURCE OF FUNDING

This research was supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg 804) and the Government of Canada Award. The authors wish to thank Kerstin Breitbach for kind and excellent support with flow cytometry; and Judy Wang, Jing-Hung Wang and Deborah A. Scollard for excellent technical assistance with ^{111}Indium-labeling and fluorescent microscopy studies.

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