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Overcoming Multidrug Resistance in Human Carcinoma Cells by an Antisense Oligodeoxynucleotide−Doxorubicin Conjugate *in Vitro* and *in Vivo*

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Abstract: Multidrug resistance (MDR), a major obstacle to successful cancer chemotherapy, may be induced by amplification of the MDR1 gene and overexpression of the P-glycoprotein (P-gp), which acts as drug efflux pump decreasing intracellular drug accumulation. In this study, an antisense oligodeoxynucleotide−-doxorubicin conjugate was used to overcome MDR in a human carcinoma-resistant cell line, both *in vitro* and *in vivo*, through downregulation of P-gp expression and mRNA levels. Compared with the unmodified antisense-oligodeoxynucleotide (AS-ODN), the conjugate markedly inhibited P-gp expression and mRNA levels. With *in vitro* treatment with the conjugate, the intracellular accumulation of doxorubicin (DOX) was increased 4.4-fold compared to treatment with DOX alone; by contrast, a 2.2-fold increase was observed when treated with AS-ODN alone. In the *in vivo* studies, it was approximately 3.5-fold higher compared to the control group treatment with DOX alone and 2.1-fold higher than found with AS-ODN. The weight of tumors formed was markedly decreased after conjugate treatment as compared to either treatments with AS-ODN or DOX alone. Furthermore, treatment with combinations of the agents appeared to be well tolerated. These results suggest that a strategy using the conjugate in combination with antitumor drugs may comprise a powerful treatment for MDR.

Keywords: Multidrug resistance; antisense oligodeoxynucleotide−doxorubicin conjugate; P-glycoprotein; KB-A-1 cells

1. Introduction

Cancer chemotherapy is often limited by the emergence of multidrug resistant (MDR) tumor cells.[1] MDR is characterized by cross-resistance to a number of structurally and functionally unrelated drugs, due to overexpression of the MDR1 gene product P-glycoprotein (P-gp). P-gp is a 170 kDa membrane glycoprotein that is capable of ATP-dependent cellular efflux of a variety of compounds across the plasma membrane. It has been shown that the overproduction of P-gp causes not only an increase in drug excretion from cells but also in a decrease in intracellular drug accumulation, which leads to a reduced intracellular drug efficacy.[2,3] Accordingly, research has been focused on how to modulate P-gp-mediated MDR. A variety of chemical inhibitors of P-gp, including verapamil, other calcium channel blockers, calmodulin inhibitors, and cyclosporin, have been shown to interfere with P-gp function and to successfully reverse the MDR phenotype *in vitro*. However, the *in vivo* efficacy of these agents has been disappointing

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due to dose-limiting toxicity and a lack of specificity.[4] Consequently, much effort is currently being directed toward developing compounds that inhibit P-gp, reverse the MDR phenotype, and sensitize tumor cells to conventional chemotherapeutic agents without the undesired toxic effects.

In the past ten years, several attempts at reversing the MDR phenotype through the use of antisense strategies have been described.[5–7] Antisense works by downregulating the gene expression by inhibiting transcription or translation by sequence-specific blinding of either DNA or RNA, respectively. The efficiency of a synthetic oligodeoxynucleotide (ODN) in regulating gene expression in living cells depends on its thermodynamic stability, resistance toward nucleases and cellular uptake. A number of studies indicate that a synthetic ODN coupled with a DNA intercalator such as acridine, naphthyl imide, psoralen or pyrene might act to increase stability.[8–10]

In a previous study we synthesized a novel antisense ODN−DOX conjugate and investigated its *in vitro* and *in vivo* pharmacokinetic properties.[11] We showed that coupling ODN with DOX at the 3′-end efficiently protects the ODN against serum and cellular 3′-exonucleases. The pharmacokinetic studies revealed that the half-life of the conjugate was about 8 h, which is four times longer than for ODN as a control. It was also shown that incubation with the conjugate decreased the level of MDR1 and the expression of P-gp in KB-A-1 cells *in vitro*.[12,13]

We addressed the question of whether the conjugate could suppress the overexpression of P-gp in KB-A-1 cells *in vivo* and could enhance DOX therapeutic activity in a KB-A-1 cancer xenograft. Using the conjugate, in combination with DOX, we were able to inhibit tumor growth and reduce the P-gp level in solid tumors in nude mice bearing the KB-A-1 cell tumor xenograft. The intracellular DOX concentration in the solid tumors, treated with or without the conjugate, was also investigated and apoptosis of the tumor cells was also determined by flow cytometric analysis. These studies raise the possibility that conjugate-targeting of MDR1 mRNA might be a useful way of restoring drug sensitivity in cancer patients that have developed MDR-mediated drug resistance.

2. Materials and Methods

2.1. Chemicals, Cell Lines and Animals. DOX was obtained from by Zhejiang Hisun Pharmacentical Co. Ltd. (Zhejiang, China). Other chemicals were obtained from commercial sources. The sequence of the AS-ODN is 5′-TCCTCCATTGCGGTCCCCTT-3′, the 30−11 region translation initiation site of the MDR1 gene resulting in MDR in KB-A-1 cells, which can downregulate the expression of the MDR1 gene.[14] The ODN with phosphate group at the 3′-end and phosphorothioate-modified ODNs (as a control) were obtained from Shanghai Bioasia Biotech Co. Ltd. (Shanghai, China). The ODN−DOX conjugate was synthesized from the 3′-end phosphate modified ODNs as previously described.[14] Lipofectamine 2000 was obtained from Invitrogen and used for the AS-ODN and ODN−DOX conjugate transfectant delivery *in vitro* study according to the supplied transfection protocol.

The doxorubicin resistant cell line (KB-A-1), a human oral epidermoid carcinoma, was generously provided by Dr. Ira Pastan and Micheal M. Gottesman (National Institutes of Health). The MDR cell line KB-A-1 was grown as adherent monolayers in flasks in DMEM with 10% fetal bovine serum and 1.0 μg/mL doxorubicin. The cells were incubated at 37 °C in a humidified atmosphere of 95% air supplemented with 5% CO_2.

Athymic male (BALB/c nu/nu) nude mice, 4−6 weeks old, were used for the KB-A-1 xenograft model. Mice were obtained from Shanghai Center of Experimental Animals,

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Chinese Academy of Sciences. The animals were provided with sterilized food and water.

2.2. Treatment of Cells with ODN−DOX Conjugate Combined with DOX. To assess the effect of the sensitivity of DOX, KB-A-1 cells were seeded in 96-well plates (Costar Corp., Cambridge, MA) in 200 μL of complete DMEM at a concentration of 1×10^4 cells/well. After incubation at 37 °C for 20−22 h, to allow the cells to attach, the medium was removed. After washing with fresh medium, the cells were treated with 0.5 μM of the conjugate or AS-ODN, wherein for control wells, only medium was added. After incubation for 24 h, the cells were washed once with medium and then exposed to DOX at doses from 0.1−20 μM for 48 h. Cell growth and viability were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[15] At the end of the incubation, the medium was removed from each well, and 100 μL of MTT solution (1 mg/mL in PBS) was added to each well. After incubation at 37 °C for 4 h, supernatants were removed and replaced by 150 μL of dimethylsulfoxide (DMSO). After incubation for 30 min at 37 °C, the optical density (OD) of each well was measured by an Automated Microplate Reader (Bio-Rad 550).

2.3. Human Carcinoma Xenograft Model and Treatment with the Conjugate Combination with DOX. The MDR cell line (KB-A-1) was established as a xenograft in athymic male nude mice. Monolayer cultures were harvested with trypsin and resuspended in PBS. About 5×10^6 cells were subcutaneously (sc) injected into the right flank of the mice. After three weeks, the tumors were aseptically dissected and tumor slurry was prepared as a single cell suspension and then was sc injected (2×10^6 cells in 0.1 mL) into the flank of each mouse.

Tumor bearing mice were randomized into groups of five, and body weights and general observations were recorded every three days. Study groups (five mice per group) included (1) untreated control; (2) nude mice injected only with DOX (2 mg/kg, intraperitoneal (ip) injection) on days 2, 4, 6, 8 and 10; (3) nude mice injected with AS-ODN (3 mg/kg, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg/kg, ip) on days 2, 4, 6, 8 and 10; (4) nude mice injected with the conjugate (1.5 mg/kg, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg/kg, ip) on days 2, 4, 6, 8 and 10; (5) nude mice injected with the conjugate (3 mg/kg, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg/kg, ip) on days 2, 4, 6, 8 and 10. All drugs were prepared in 0.1 mL of aseptic water 1 h before use. The animal weight and tumor volume were measured every 4 days. The tumor volume was measured in two perpendicular diameters (length and width). Caliper measurements of tumors were converted into mean tumor weight (g) using the formula (1/2)[length (cm)] × [width (cm)]^2. A plot of tumor growth was drawn of tumor weight against time of implantation. The mice were killed at day 21, and tumor weights were measured. The rate of inhibition (IR) was calculated according to the formula IR = [1 − (tumor weight in the drug treatment group)/(tumor weight in the control group)] × 100%. All animals were euthanized on day 21 from the start of treatment, and tumors were removed, weighed and finally stored at −70 °C.

2.4. Detections of the DOX Concentrations in the Cells, Tumors and Normal Tissues. The intracellular DOX concentration was determined by HPLC analysis.[16] Cells from exponentially growing cultures were harvested and dispensed into 24-well culture plates in 1000 μL of medium at a concentration of 10^5 cells/well for the KB-A-1 cell line. The cells were exposed to 10 μg/mL DOX and 2 μg/mL AS-ODN or 2 μg/mL conjugate, while the control was treated with 10 μg/mL DOX alone. After incubation for 24 h, the cells were washed twice with cold PBS, resuspended in 0.5 mL of distilled water and then frozen and thawed repeatedly. After mixing thoroughly, the mixtures were centrifuged for 30 min at 10,000 rpm. The DOX content in the supernatant was then determined by RP-HPLC.

The mobile phase consisted of a mixture of 5 mM phosphoric acid, methanol, isopropanol, acetonitrile (8:7:3:2, by volume) and was vacuum filtered through 0.45 μm nylon filters and sonicated before use. The flow rate of the mobile phase was 0.7 mL/min. Fluorescence detection was performed using excitation and emission wavelengths of 495 and 560 nm, respectively. DOX concentrations were determined using a DOX standard curve, and measurements were performed in duplicate and reported as mean concentrations.

A portion of each frozen sample was processed for measurement of tumor and normal tissue DOX levels as follows.[17] The tumor was homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) in 20 mM KH_2PO_4 buffer (pH 3.8; 1:5, by volume). Forty microliters of internal standard (10 μg/mL DOX), 30 μL of 20 mM KH_2PO_4, 60 μL of 33% silver nitrate, and 390 μL of 80% acetonitrile in water were added to 300 μL of tumor homogenate. Samples were then vortexed and centrifuged for 10 min at 3000 rpm to precipitate protein, and 50 μL aliquots of the supernatant were analyzed by HPLC and the DOX concentrations obtained as described above.

2.5. Total RNA Extraction and mRNA Detection by RT-PCR. Total RNA extraction and mRNA detection by RT-PCR were carried out as described elsewhere.[12,13] The total RNA from KB-A-1 cells was isolated as follows. Cells were grown to 1×10^5 per well in 24-well culture plates. 200 μL of TRIzol was added after removal of the culture medium. Cell lysate was then passed several times through a pipet and placed into a 1.5 mL polypropylene tube. Eighty microliters of chloroform was added, and the tube was shaken

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violently for 1 min and then incubated for 3 min at room temperature, following which it was centrifuged at 12000*g* for 15 min at 4 °C. The aqueous phase was then transferred to a fresh tube and mixed with 200 μL of isopropanol followed by incubation at room temperature for 10 min, following which it was centrifuged at 12000*g* for 10 min at 4 °C. The supernatant was discarded, the pellet was washed with 500 μL of 75% alcohol, and then the mixture was vortexed and centrifuged at 7500*g* for 5 min at 4 °C. The pellet obtained was dried for 10 min at 60 °C and then redissolved in distilled water. Each sample was stored at −80 °C until required for the RT-PCR assay.

For quantitative RT-PCR, fluorescent hybridization probes and TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with the ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were purchased from CASarray Co., Ltd. (Shanghai, China). TaqMan probes for MDR1 was purchased from PE Applied Biosystems. The primers and TaqMan probes used were as follows: the sequence of the forward primer for MDR1 mRNA was 5′-GTCTACGTTCGTAATGCTGACGT-3′, and that of the reverse primer was 5′-TGTGATCCACGGA-CACTCCTAC-3′; the TaqMan probe was 5′-CGCTG-GTTTCGATGATGGAGTCATTG-3′. The forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCGGAGT-3′, and the reverse primer was 5′-GAAGATGGTGATGG-GATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGT-TCTCAGCC-3′. Conditions for one-step RT-PCR were as follows: 2 min at 50 °C (stage 1, reverse transcription), 10 min at 95 °C (stage 2, RT inactivation and AmpliTaq Gold activation) and then 45 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C (stage 3, PCR). The expression of MDR1 was then quantified. Briefly, the 25 μL of reaction mixture contained 1 × TaqMan buffer A, 5.5 μM MgCl2, 400 μM dUTP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 0.01 U/mL AmpErase UNG, 0.025 U/mL AmpliTaq Gold DNA Polymerase, 200 nM of each forward and reverse primer, 100 nM TaqMan probe (PE Applied Biosystems) and 1 μL of RT product. The PCR process was performed first at 95 °C for 10 min and then at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s with the last three steps repeated for 50 cycles. The relative increase in the emission of fluorescence was monitored in real time using a sequence detector (ABI prism 7700 sequence detector Applied Biosystems). The value of the cycle number (CT) is linearly correlated with the logarithmic value of the amount of the mRNA levels of MDR1 and GAPDH. The mRNA levels of MDR1 are expressed as concentrations relative to GAPDH mRNA, and the values are given as the mean ± SD.

2.6. Western Blot Analysis of P-Glycoprotein Levels in the Cells and Tumors. Western blot analysis of P-glycoprotein levels in the cells was carried out as we have previously described.[12,13] KB-A-1 cells were cultured in 10 cm diameter culture dishes and grown to 60% confluency. The medium was removed and substituted with fresh culture medium added with the conjugate or the AS-ODN. When the cells reached 90% confluency, they were washed with ice-cold PBS, harvested using a rubber tipped glass rod and finally lysed in 500 μL of buffer A (20 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate, 50 μM NaF, 20 μM leupeptin, 10 μM β-mercaptoethanol, 2.5 mM sodium pyrophosphate, 0.2 mM phenylmethanesulfonyl fluoride) plus 1% Triton-100 at 0 °C for at least 30 min. After sonication for 5 min at 0 °C, the suspensions were centrifuged at 4000*g* for 15 min at 4 °C. The supernatant was collected and centrifuged at 100000*g* for 60 min at 4 °C. Each pellet was resuspended in 200 μL of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate, 50 μM NaF, 20 μM leupeptin, 10 μM β-mercaptoethanol, 2.5 mM sodium pyrophosphate, 0.2 mM phenylmethanesulfonyl fluoride, 0.25 M sucrose) and reserved at −80 °C until required for Western blot analysis.

Cell membrane protein (15 μg/mL) were analyzed using an 8% SDS−polyacrylamide gel with a 5% stacking gel. The resolved proteins were transferred to a nitrocellulose membrane from the gel. The nitrocellulose blots were blocked for 4 h at 37 °C using phosphate buffer saline and Tween (PBST) buffer (0.1% Tween-20) containing 3% bovine serum albumin (BSA). Next, after washing with PBST twice, the blots were incubated overnight with C219 anti-P-170 monoclonal antibody (Invitrogen, Carlsbad, CA) at a concentration of 1.0 μg/mL in a PBST containing 2% BSA. Following the washing, as described above, the blots were incubated with a second antibody (peroxidase-conjugated goat antimouse IgG, Calbiochem, Canada) using a 1:2500 dilution for 60 min at room temperature. Then the blots were washed again and visualized with enhanced chemiluminescence detection and imaged onto Kodak X-OMAT film. The relative expressions were calculated according to the reference bands of β-actin.

Two hundred milligrams of tumor tissue was homogenized in 2 mL of ice-cold PBS, and 1 mL of tissue homogenate was transferred to a fresh tube, and then centrifuged at 1000 rpm for 5 min at room temperature. Each pellet was resuspended with 1 mL of lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM sodium orthovanadate, 50 μM NaF, 20 μM leupeptin, 10 μM β-mercaptoethanol, 2.5 mM sodium pyrophosphate, 0.2 mM phenylmethanesulfonyl fluride) plus 1% Triton-100, and then was incubated at 0 °C for 30 min with some vortexing. Western blot analysis was carried out as described above. The protein concentration was determined by chemiluminescence using Coomassie brilliant blue G-250 with bovine serum albumin, and the standard curve was constructed.

2.7. Flow Cytometry Assay. Fresh tumor tissue was rapidly removed, weighed, and placed into 10 mL of ice-cold PBS containing 0.2% bovine serum albumin (BSA) (Sigma), 10 mM EDTA, and 10 mg/mL deoxyribonuclease I (Sigma). The tissue was then disrupted using a glass homogenizer and passed through a 40 μm nylon cell strainer (Becton Dickinson). The suspension was centrifuged at 500*g* for 10 min at room temperature. The pellet was resuspended

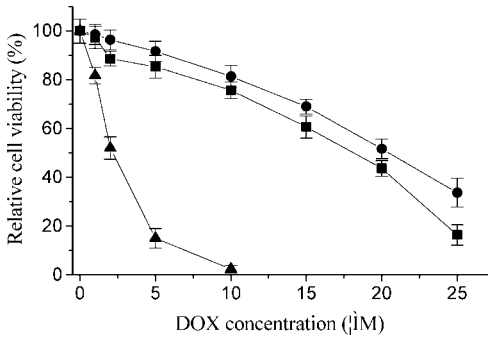

Figure 1. Drug sensitivity effect of the conjugate or AS-ODN in KB-A-1 cells. Cells were treated with the conjugate (▲), or AS-ODN (■) at a dose of 0.5 μM for 24 h, and then exposed to DOX (1.0−25 μM) for 48 h. The control cells (●) were exposed to DOX alone (same dose and exposure time). Cell viability was measured by MTT assay. Relative cell viability = [(mean OD of one group)/(mean OD of the control)] × 100%. Results are means ± SD of four different experiments.

in 500 μL of PBS with BSA and transferred into a fresh tube. The cells obtained were fixed with ice-cold 70% ethanol in PBS at 4 °C for 8 h, then incubated with RNase (20 μg/mL) for 30 min at 37 °C and labeled with propidium iodide (50 μg/mL). DNA content was measured using a FACS Calibur cytometer (Becton Dickinson). The multicycle software package (CELLQUEST software, Becton Dickinson) was used to produce histograms of DNA content frequency. Subdiploid DNA peaks were quantified from the DNA content data to measure apoptosis.

2.8. Statistical Analysis. Data are expressed as the mean ± one standard deviation (SD) and analyzed by the ANOVA and *posthoc* test. *P*-values below 0.05 were regarded as statistically significant.

3. Results

3.1. Reversal of MDR in KB-A-1 Cells Treated with the Conjugate in Vitro. The ability of the conjugate to reverse DOX cytotoxicity in KB-A-1 cells and therefore to reverse MDR was assessed using experiments in which KB-A-1 cells were first exposed to the conjugate and then to DOX. The results shown in Figure 1 reveal that only when KB-A-1 cells were treated with DOX alone at the highest dose (15 μM) was cell growth inhibited (approximately 28% inhibition, compared to control). When treated with the conjugate, a significant increase of DOX cytotoxicity was observed, the inhibition of the cell growth being ~47% at the lower DOX dose (2.0 μM). By contrast, only a slight effect was observed when treated with the mixture of the AS-ODN (0.5 μM) and DOX (0.5 μM). DOX IC_{50} values (the dose of DOX that caused 50% of cell growth inhibition) were calculated from the percentage of growth inhibition induced by the different treatments on KB-A-1 cells. The DOX IC_{50} value for the conjugate was 2.2 μM. By contrast, the DOX IC_{50} value for the AS-ODN mixed with DOX was 16.8 μM, and for DOX alone the value was 21.5 μM. The

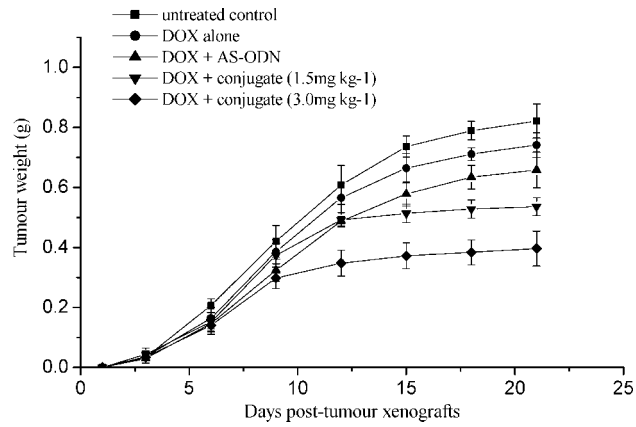

Figure 2. Antitumor efficacy of DOX with or without the antisense conjugate or the antisense ODN against human carcinoma cancer (KB-A-1 cells) xenografts in nude mice. KB-A-1 tumors were implanted in the flanks of the mice. Treatments: (■) untreated control; (●) nude mice injected only with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (▲) nude mice injected with AS-ODN (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (▼) nude mice injected with the conjugate (1.5 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10. (◆) nude mice injected with the conjugate (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10. Data are expressed as means ± SE ($n = 5$).

reversal index of the conjugate (equal to the DOX IC_{50} value for DOX alone/the DOX IC_{50} value for the conjugate) was nearly 10. The reversal index of the AS-ODN was 1.3. These results demonstrate that the conjugate displayed a strong reversal effect on MDR.

3.2. Reversal Effect of the Conjugate on MDR in Xenografts. In the *in vivo* model, established by the nude xenograft, when MDR tumors were treated with the conjugate, a suppression of tumor growth was observed. Figure 2 shows the tumor growth in the nude xenografts of KB-A-1 cells with time. Tumor growth was much slower for the group given DOX and the conjugate compared with the DOX alone groups. The tumor growth was slower in the higher dosage group in contrast to the lower dosage group.

The tumor weights for the control group, for DOX alone, or DOX in combination with AS-ODN, and the coadministration groups at day 21 after implantation are shown in Figure 3. The results show that DOX alone had no significant anticancer action in the nude mice xenografts of KB-A-1 cells, and only had a 14.6% inhibition rate; however, the combination of DOX and the conjugate significantly inhibited the growth of the xenograft and the inhibition rate (versus the control weight of tumor) was 56.7% (high dose) and 49.4% (low dose). For the combination of DOX with AS-ODN, the inhibition rate (versus the control weight of tumor) was 26.9%. The result demonstrates that the conjugate could reverse MDR *in vivo*.

Importantly, the regimen of the combination of DOX and the conjugate did not cause any death in our *in vivo* models.

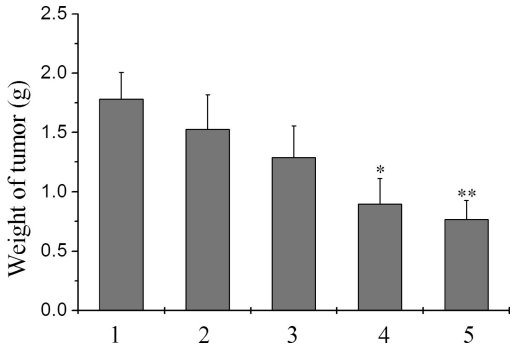

Figure 3. Weight of tumors in nude mice bearing human carcinoma cancer (KB-A-1 cells) xenografts. Tumors were implanted in the flanks of the mice. The mice were killed at day 21 after implanting, and tumor weights were measured. Treatments: (1) untreated control; (2) nude mice injected only with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (3) nude mice injected with AS-ODN (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (4) nude mice injected with the conjugate (1.5 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (5) nude mice injected with the conjugate (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10. Data are expressed as means ± SE ($n = 5$). Significant differences from the control group are indicated by * and ** ($p < 0.05$ and $p < 0.01$, respectively, ANOVA and *posthoc* test).

The average weight of the animals was higher in the postexperiments than in the pre-experiments in the combination group; however, the increased animal weight was not significantly different among the different groups (Figure 4). The difference of animal weight between for the lower concentration of the antisense ODN conjugate (1.5 mg/kg) treatment and the higher concentration (3.0 mg/kg) was apparent in the pre-experiments. The lack of a weight difference between the groups is consistent with the regimen having no increased toxic side effects, suggesting that the toxicity for coadministration of DOX and the conjugate under the test dose was tolerable.

3.3. Intracellular DOX Concentration in KB-A-1 Cells. When KB-A-1 cells were exposed to 2 μg/mL AS-ODN, or 2 μg/mL conjugate combined with 10 μg/mL DOX for 24 h, the intracellular concentrations of DOX were 0.9 ± 0.02 and 3.2 ± 0.04 μg/mL, respectively, compared with untreated controls exposed to 10 μg/mL DOX alone, in which the intracellular concentration of DOX was 0.5 ± 0.01 μg/mL (see Table 1). The results show that the intracellular concentration of DOX in KB-A-1 cells increased after treatment with AS-ODN and the conjugate. When treated with 2 μg/mL conjugate alone for 24 h, the intracellular DOX concentration was 0.03 ± 0.01 μg/mL, as shown in Table 1. The data indicate that the increased intracellular doxorubicin accumulation in KB-A-1 cells did not result from a contribution of the DOX moiety of the conjugate.

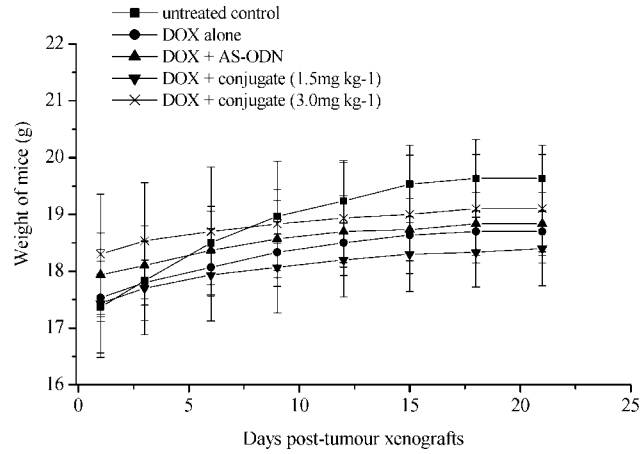

Figure 4. Weight−time profiles of the mice bearing human carcinoma cancer (KB-A-1 cells) xenografts treated with DOX combination with or without the antisense conjugate or the antisense ODN. KB-A-1 tumors were implanted in the flanks of the mice. Treatments: (■) untreated control; (●) nude mice injected only with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (▲) nude mice injected with AS-ODN (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (▼) nude mice injected with the conjugate (1.5 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10. (×) nude mice injected with the conjugate (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10. Data are expressed as means ± SE ($n = 5$).

Table 1. The intracellular concentration of doxorubicin for 10^5 KB-A-1 cells[a]

group[b]	doxorubicin added (μg/mL)	intracellular doxorubicin (μg/mL)	RM
1	2.0 μg/mL conjugate alone	0.03 ± 0.01	
2	10	0.5 ± 0.01	
3	10	0.9 ± 0.02	1.8
4	10	3.2 ± 0.04	6.4

[a] The intracellular concentration of doxorubicin was determined by HPLC, in the presence or absence of AS-ODN and the conjugate. Each value represents the mean ± standard deviation (SD) of four independent experiments. The reversal multiple (RM) of each drug was calculated by (concentration of DOX in the combined group)/(concentration of DOX in the doxorubicin alone group). [b] (1) 2.0 μg/mL conjugate alone; (2) 10 μg/mL doxorubicin alone; (3) 10 μg/mL doxorubicin combined with 2.0 μg/mL AS-ODN; (4) 10 μg/mL doxorubicin combined with 2.0 μg/mL conjugate.

3.4. DOX Concentration in Tumors and Normal Tissues. The concentration of DOX in the tumors and in normal tissues is shown in Table 2. The results demonstrate that the DOX concentration in the tumor group treated with a high dose of conjugate was higher than the others. Thus, it was approximately 4.5-fold higher than the group treated with DOX alone and 2.3-fold higher than the group treated with AS-ODN. The data indicate that the conjugate showed a potent and dose-dependent drug accumulation effect in nude mice tumors, consistent with our *in vitro* results.

Table 2. The Concentration of DOX in Solid Tumors and Normal Tissues of Nude Mice Bearing Human Carcinoma Cancer (KB-A-1 Cells) Xenografts[a]

group[b]	tumor doxorubicin (ng/mg)	liver doxorubicin (ng/mg)	kidney doxorubicin (ng/mg)	heart doxorubicin (ng/mg)
1	4.8 ± 0.8	6.1 ± 0.8	4.3 ± 0.7	6.9 ± 1.1
2	9.2 ± 1.4	6.3 ± 1.1	5.0 ± 1.8	8.2 ± 2.1
3	17.1 ± 2.3	6.8 ± 1.5	5.8 ± 1.3	8.6 ± 2.5
4	21.6 ± 4.1	7.1 ± 1.9	6.3 ± 2.0	8.8 ± 2.9

[a] Tumors were implanted in the flanks of the mice. The mice were killed at day 21 after implanting, and then the tumors were collected and HPLC performed. Data are expressed as means ± SE ($n = 5$). Each value represents the mean ± standard deviation (SD) of four independent experiments. [b] Treatments: (1) nude mice injected only with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (2) nude mice injected with AS-ODN (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (3) nude mice injected with the conjugate (1.5 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10; (4) nude mice injected with the conjugate (3 mg kg^{-1}, ip) on days 1, 3, 5, 7, 9 and injected with DOX (2 mg kg^{-1}, ip) on days 2, 4, 6, 8 and 10.

Some MDR reversal agents exhibit severe toxicity and enhanced antitumor drug-induced toxicity in normal tissues, therefore the effect of the conjugate on the accumulation of DOX in the liver, heart and kidneys in tumor-bearing mice was also examined in this study (see Table 2). The DOX concentration in these normal tissues in the group treated with the conjugate was not significantly different from the group treatment with DOX alone. This suggests that the conjugate did not increase DOX-induced toxicity in normal tissues in tumor-bearing mice.

3.5. Effect on the Expression of MDR1 mRNA in KB-A-1 in Vitro. mRNA expression levels were quantified by reverse transcriptase-coupled polymerase chain reaction (RT-PCR) with an endogenous standard after cells were exposed to drugs for 24 h. Figure 5 shows results of electrophoresis of the amplified PCR products of MDR1 mRNA isolated from cells treated with AS-ODN and the conjugate, respectively. The data for real-time PCR indicate that both the AS-ODN and the conjugate might suppress the expression of MDR1 message in KB-A-1cells by more than 25%, as shown in Figure 5b. The relative expression ratio of MDR1/GAPDH mRNA is 59.3% for the conjugate group and 74.5% for the AS-ODN group, respectively, as compared with the control group. However, the conjugate showed 12% greater effect compared with free AS-ODN on the expression of MDR1 mRNAs. The levels of GAPDH mRNA were essentially unchanged in cells receiving different treatments, confirming the specificity of the effects induced by the conjugate.

3.6. Effect of the Conjugate on KB-A-1 Cell Expression of P-gp in Vitro and in the Tumors. With respect to the MDR phenotype, the KB-A-1 cell line has been demonstrated to overexpress P-glycoprotein. To determine the effects of the various treatments on P-gp expression in the KB-A-1 cells and xenograft tumors, a Western blot analysis was performed. When treated with the conjugate

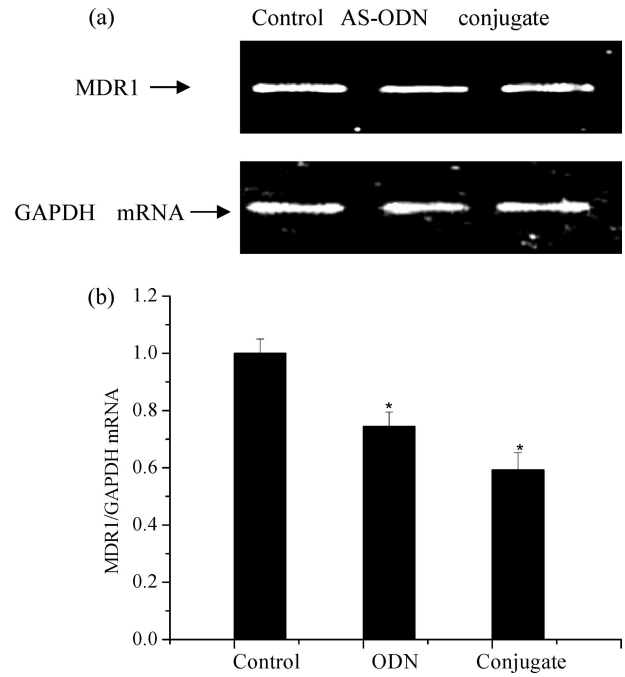

Figure 5. (a) Expression of MDR1 mRNA in KB-A-1 cells treatment with the conjugate or the AS-ODN (0.5 μM) by RT-PCR analysis. Cell treatment with DOX alone acts as a control. (b) The levels of MDR1 mRNA in KB-A-1 cells were measured by real-time quantitative RT-PCR. Each column represents the mean SD of three experiments, as percentages of the MDR1/GAPDH mRNA levels in the control group. *$P < 0.01$, significantly different from the control.

(0.5 μM), the P-gp expression in KB-A-1 cells was significantly decreased as compared to the AS-ODN (0.5 μM) treatment alone, as shown in Figure 6a. The same results were obtained in the tumors (see Figure 6b). A concentration-dependent inhibition of the MDR1 mRNA level in the KB-A-1 cells incubated with the conjugate was also observed, while AS-ODN at the same concentration exerted only a slight affect on the MDR1 mRNA level (see Figure 5). The results indicate that the decreased P-gp expression resulted directly from an antisense-mediated specific inhibition of MDR1 mRNA.

3.7. Effect of the Conjugate on the DOX-Induced Apoptosis in the Tumors. A flow cytometric assay was carried out to examine the effect of the conjugate on the DOX-induced apoptosis in the mice tumor cells. Apoptosis for the group treated with DOX alone was 32.5%, 2.8-fold greater than that for the control, as shown in Figure 7. Apoptosis in the group treated with AS-ODN combined with DOX was 48.6%, 1.5-fold greater compared with the DOX alone treatment group. However, apoptosis in the group treated with the high dose of the conjugate combined with DOX was 65.5%, 2.0-fold greater compared with the DOX alone treatment group. These results indicate that the conjugate was able to increase apoptosis in the tumor cells.

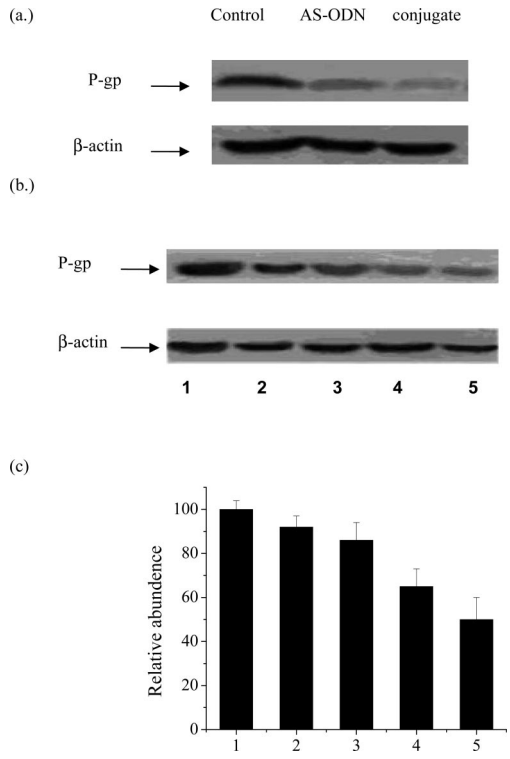

Figure 6. (a). P-gp levels were assessed with Western blot performed at the end of the AS-ODN (0.5 μM) or the conjugate (0.5 μM) treatment in KB-A-1 cells using C219 monoclonal antibody. (b). P-gp levels were assessed by Western blot analysis in the tumors treated with the conjugate, or AS-ODN in combination with DOX, using C219 monoclonal antibody. 1: Control. 2: DOX alone. 3: DOX + the AS-ODN. 4: DOX + the conjugate (low dose). 5: DOX + the conjugate (high dose). (c) Quantification of Western blot analysis of relative abundance of P-gp expression *in vivo*.

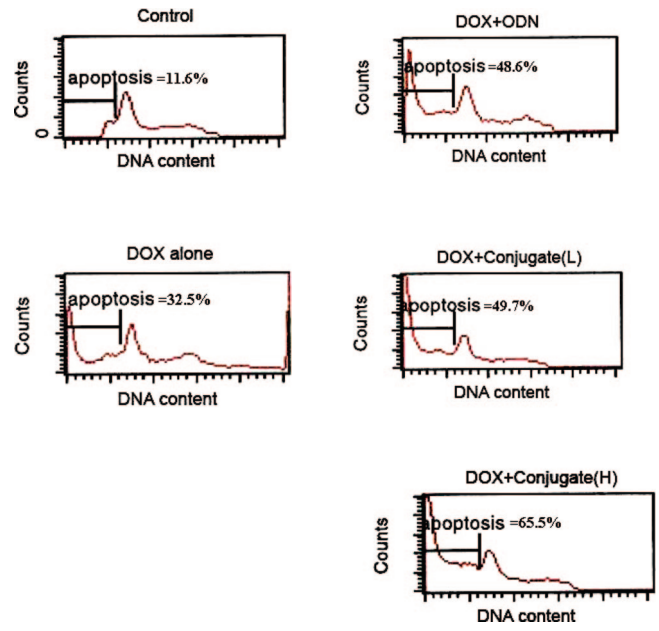

Figure 7. Analysis of apoptosis of the tumor cells using flow cytometry. Tumors tissues were processed to isolate cells, and the cells were then stained with propidium iodide and analyzed in a FACScan flow cytometer. Apoptosis-associated subdiploid peaks appeared on the flow cytometry histograms.

4. Discussion

The use of AS-ODN is normally designed to target mRNA and is considered to be an inhibitor of certain proteins as a gene-silencing strategy. Compared with other similar approaches, such as ribozyme and RNAi technology, studies on the use of AS-ODN have demonstrated its feasibility, potency and safety in the clinical setting. The first AS-ODN drug, formivirsen, has come into the market successfully, and a great number of AS-ODN drugs are now undergoing clinical trails.[19]

The development of MDR in cancer cells still represents one of the major reasons for failures in anticancer chemotherapy.[1] Therefore the major objective of the present study was to attempt a modulation and eventual reversal of MDR in a human carcinoma-resistant cell line, both *in vitro* and *in vivo*, using the AS-ODN−DOX conjugate targeted to the MDR1 gene. For this study KB-A-1 cells were used, which overexpress P-gp and exhibited resistance to DOX and other anticancer drugs related to the MDR phenotype. Different AS-ODNs have been reported to selectively downregulate P-gp expression,[20,21] suggesting that their use might have therapeutic potential in cancer treatment after the emergence of the drug-resistant phenotype. Unmodified AS-ODNs have a short half-life in biological systems; we therefore used a conjugate that was covalently linked with DOX at the 3′-end on the AS-ODN as the antisense agent. In our previous studies, we found that the AS-ODN-linked DOX had a markedly improved resistance to endonuclease in cell culture conditions, and strongly stabilized the complex formed from the AS-ODN and its complementary sequence.[14] Moreover, pharmacokinetic studies *in vivo* show that the half-life of the conjugate is 8 h, which is four times longer than for AS-ODN alone.[11]

In the present study, a marked downregulation of P-gp expression and MDR1 was observed by using the combination of DOX with the conjugate (0.5 μM) *in vivo* (Figure 6b). This observation is consistent with previous *in vitro* studies (Figure 5, Figure 6a). The results indicate that the decrease of P-gp expression directly resulted from an

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antisense-mediated specific inhibition of MDR1 mRNA. The difference in P-gp expression and MDR1 mRNA levels in KB-A-1 cells, between treatment with the conjugate and with the AS-ODN, is due to the difference of the stability and the intracellular concentration in the cells. It is also due to the improved stability of the conjugate and the fact that the intracellular concentration of the conjugate was higher than that of the AS-ODN (data not shown). In addition, the increased binding affinity of the conjugate for the complementary sequence may contribute to the difference.[8,9] P-gp, which functions as a pump, is capable of removing the drug from the cytoplasm. Therefore, the reduced P-gp levels may have been induced by the increased DOX concentration in the cells (Table 1) and in the tumors (Table 2). *In vivo*, we also observed that there was no significant difference in the DOX concentration in the normal tissues, namely in the liver, kidney and heart, between the combination and DOX alone treatment groups (Table 2). These results suggest that the conjugate, under the test dosage, could potentiate the antitumor effect of DOX without exhibiting toxicity to the normal tissues in tumor-bearing mice.

The observed *in vitro* reversal of MDR correlates well with the results obtained *in vivo* using nude mouse solid tumor xenograft models. *In vivo*, the conjugate given in combination with DOX was able to sensitize the resistant KB-A-1 cells to the effect of DOX and thus to inhibit the growth of the tumors in the mice. The inhibition rate of the conjugate was 2.1-fold higher than that of the AS-ODN alone (Figure 2). This result indicates that the enhanced antitumor activity of DOX was significantly increased in the tumor-bearing mice by the conjugate as compared with AS-ODN. Although the concentration of DOX could be increased by the treatment of the conjugate, for the resistant xenograft, this would not be expected to markedly affect the tumor growth under the test dosages. This marked change in tumor growth might be a consequence of the suppressed MDR expression by conjugate, which sensitizes the resistant KB-A-1 cells to the effect of DOX. DOX, as a potent cytotoxic agent, can induce apoptosis of tumor cells *in vitro* and *in vivo*.[22] Additional evidence of an enhanced antitumor effect of DOX by the conjugate is that the conjugate promoted DOX-induced apoptosis in the resistant KB-A-1 derived tumors (Figure 7). The effects would therefore appear to be caused by an accumulation of the DOX concentration in the tumors.

In conclusion, the AS-ODN−DOX conjugate could chemosensitize resistant tumor cells to DOX *in vitro* and *in vivo* through a downregulation of P-gp expression and mRNA levels in the resistant cells, and thus increase the accumulation of the intracellular DOX in the cells and in the tumors. The conjugate could reverse MDR *in vivo* and also did not increase toxic side effects under this dose. Therefore, the conjugate may be an efficient and suitable modulator of MDR not only *in vitro*, but also *in vivo*.

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