

# Reversal of doxorubicin resistance by adenovirus-mediated transfer of cyclooxygenase-2 antisense in multidrug-resistant MCF-7 cells

## Research Article

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**Abbreviations:** Adenomatous Polyp Prevention on Vioxx, (APPROVE); adenovirus vector expressing COX-2 antisense, (Ad-COX-2 AS); cyclooxygenase-2, (COX-2); deoxynucleotide triphosphate mixture, (dNTP); doxorubicin-resistant breast cancer cell line, (MCF-7/DOX); enzyme-linked chemiluminescence, (ECL); ethylenediaminetetraacetic acid, (EDTA); multidrug-resistance, (MDR); phosphate buffered saline, (PBS); phosphate buffered saline-Tween, (PBS-T); plaque forming units, (PFU); radioimmunoprecipitation, (RIPA); sodium dodecylsulfate, (SDS)

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## Summary

Increased expression of cyclooxygenase-2 (COX-2) has been implicated in many human malignancies, and it is associated with tumor progression, angiogenesis, inhibition of apoptosis, and development of multidrug-resistant (MDR) phenotype. Both selective and nonselective COX-2 inhibitors have been used in clinical trials as adjuvant therapy—too often at the expense of increased systemic toxicity. In the current study, we evaluated whether adenovirus vector expressing COX-2 antisense (Ad-COX-2 AS) has therapeutic potential to modulate the drug resistance and the expression of MDR gene MDR1, antiapoptotic gene Bcl-2, and cell cycle regulating gene cyclin D1 in doxorubicin-resistant breast cancer cell line (MCF-7/DOX). Our data show that MCF-7/DOX cells are more efficiently transduced by adenovirus compared with the parental MCF-7 cell line. Transduction of MCF-7/DOX cells with Ad-COX-2 AS (multiplicity of infection [MOI] of 10, 20, 50, plaque forming units [PFU]/cell) reduced the IC<sub>50</sub> value of doxorubicin by 13-, 19-, and 20-fold, respectively, compared with Ad-LacZ–transduced cells ( $P < 0.05$ ). Furthermore, Ad-COX-2 AS downregulates the expression of the MDR1, the antiapoptotic protein Bcl-2, and the cell cycle regulating protein cyclin D1 in MCF-7/DOX cells. In conclusion, MCF-7/DOX cells are more efficiently transduced with Ad-COX-2 AS compared with parental MCF-7 cells. Transduction of MCF-7/DOX cells with Ad-COX-2 AS reverses their resistance to doxorubicin. The promising efficacy of Ad-COX-2 AS in reversing drug resistance deserves further investigation.

## I. Introduction

There is considerable evidence to suggest that the expression of cyclooxygenase-2 (COX-2) plays an important role in the development of many human malignancies, and that it confers a survival advantage that eventually facilitates tumor development, progression, angiogenesis, and resistance to chemotherapeutic agents

(Chow et al, 2005; Tari et al, 2005; Zatelli et al, 2005; Mazhar et al, 2006; Raspollini et al, 2006). The effects of COX-2 overexpression in cancer cells are mediated by interactions with downstream effectors of COX-2. Recent reports suggest that expression of the COX-2 enzyme upregulates expression of MDR1/P-glycoprotein (P-gp), an exponent of resistance to anticancer drugs (Ratnasinghe

et al, 2001; Patel et al, 2002; Ziemann et al, 2002; Sorokin 2004; Surowiak et al, 2005), suggesting that COX-2 is involved in inducing the chemoresistance phenotype. Accordingly, numerous *in vivo* and *in vitro* studies indicate that COX-2 inhibitors (eg, coxibs) enhance the efficacy of various anticancer therapy methods (Gasparini et al, 2003).

COX-2 plays a role not only in the growth of cancer cells but also in inhibiting the apoptosis pathway. Various experimental studies have shown a positive correlation between the expression of COX-2 and the inhibition of apoptosis (Meric et al, 2006). COX-2 overexpression is associated with increased amounts of the antiapoptotic protein Bcl-2 (Tsujii and DuBois, 1995). Furthermore, after a pre-exposure of tumor cells to prostaglandin E<sub>2</sub> *in vitro*, a 4- to 5-fold increase in the levels of the antiapoptotic protein Bcl-2 has been observed, whereas the level of the Bax proapoptotic protein remained unchanged (Sheng et al, 1998). In addition to its antiapoptotic effect, COX-2 enhances tumor cell proliferation by inducing the expression of cyclin D1 (Wu et al, 2004; Patel et al, 2005; Zhang et al, 2006). Taken together, these data show a causal relationship between the expression of COX-2 and the inhibition of apoptosis, stimulation of cell progression, and development of drug resistance in tumor cells. Therefore, selective COX-2 inhibitors have emerged as potentially important chemopreventive and therapeutic agents in cancer treatment (Becerra et al, 2003; Chow et al, 2005; Mazhar et al, 2006). Despite considerable evidence suggesting a therapeutic role for COX-2 inhibitors in the chemoprevention and possible treatment of breast cancer, the available COX-2 inhibitors are not optimal compounds, and their use is associated with serious cardiovascular effects (Becerra et al, 2003; Dogne et al, 2006; Meric et al, 2006). In fact, the recent withdrawal of rofecoxib due to increased cardiovascular and thrombotic risk during the Adenomatous Polyp Prevention on Vioxx (APPROVe) trial has underscored new safety concerns about this class of drug. Thus, development of novel strategies that target COX-2 inhibitors selectively into tumor cells, while avoiding systemic toxicity, will be an invaluable addition in cancer management. The application of gene therapy approaches that can be adapted to transcriptional or transductional targeting of particular tissues or cell types, or certain cancer cells, represents a novel therapy that is distinct from traditional pharmaceutical treatment modalities. Antisense oligonucleotides to specific cellular RNA have shown great promise in both research and clinical studies as these sequence-specific agents are able to modulate the expression of targeted genes. In principle, the most important feature of antisense oligonucleotides is their ability to base-pair with the target RNA and either block its translation or facilitate its destruction by RNase H, an enzyme that destroys RNA in a DNA/RNA duplex (Tyson-Capper and Europe-Finner, 2006). In this study, we have examined the potential therapeutic effect of adenovirus vector expressing COX-2 antisense (Ad-COX-2 AS) on modulating the expression of multidrug-resistance (MDR) gene MDR1, antiapoptotic gene Bcl-2, and cell cycle regulating gene cyclin D1, as well as the

chemosensitivity to doxorubicin in a doxorubicin-resistant breast cancer cell line (MCF-7/DOX).

## II. Materials and Methods

### A. Cell culture and cell lines

The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (Rockville, Md). MCF-7/DOX cells, a human breast adenocarcinoma cell line highly resistant to doxorubicin and overexpressing the MDR protein P-gp170 (Chen et al, 2002), was a kind gift from Dr. Kapil Mehta at the University of Texas M.D. Anderson Cancer Center, Houston, Texas. The cell lines were maintained at 37°C in a 95% humidified, 5% CO<sub>2</sub> atmosphere in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin and streptomycin (Gibco BRL, Life Technologies, Long Island, NY). To maintain the resistance phenotype, the MCF-7/DOX cells were grown in the presence of 1 µg/mL doxorubicin (Chen et al, 2002).

### B. Recombinant adenoviral vector and *in vitro* transduction

The replication-deficient Ad-COX-2 AS under the control of the cytomegalovirus promoter was a generous gift from Dr. Chuan-Ming Hao (Vanderbilt University School of Medicine, Nashville, Tenn). An adenovirus expressing a marker gene coding for bacterial β-galactosidase (Ad-LacZ) was a kind gift from Dr. Savio Woo (Mount Sinai School of Medicine, New York, NY). Large-scale production of Ad-COX-2 AS and Ad-LacZ was performed as we described previously (Al-Hendy and Auersperg, 1997), with a typical batch yield of  $2 \times 10^{10}$  PFU/mL. For the *in vitro* transduction, MCF-7/DOX cells were grown as described above until they reached 70% to 80% confluence. Then, the cells were transduced with the Ad-LacZ reporter vector or Ad-COX-2 AS at the desired MOI for 4 hours in transduction media as we described previously (Al-Hendy and Auersperg, 1997). The transduction media were removed and replaced with maintenance media. The transduction efficiency was determined in Ad-LacZ-transduced cells by staining for Ad-LacZ expression with b-galactosidase staining kit (Stratagene, La Jolla, Calif), according to the manufacturer's protocol. To study the effect of the Ad-COX-2 AS on MCF-7/DOX cells, the cells were transduced with different MOI (10, 20, 50, and 100 PFU/cell) of Ad-COX-2 AS or Ad-LacZ as a negative control.

### C. Cytotoxicity assay

MCF-7/DOX cells transduced with the indicated virus were plated in 6-well plates ( $5 \times 10^5$  cells/well) and allowed to attach for 12 hours. The growth media were replaced with fresh media containing different concentrations of doxorubicin (0.01–20 µM). After 72 hours, the media were removed from the wells, and the cells were washed twice with phosphate buffered saline (PBS). The number of viable cells per well was determined in triplicate using a fluorometric assay with Hoechst 33258 (bisbenzimidazole), as described previously (Houston et al, 2003). A similar set of experiments was performed with the Trypan blue (Sigma Chemical Co, St. Louis, Mo) exclusion test. The concentrations of doxorubicin required to inhibit growth by 50% (IC<sub>50</sub> values) were calculated from the cytotoxicity curves using GraphPad Prism Software (GraphPad Software Inc, San Diego, Calif). The fold-reversal of doxorubicin resistance was calculated by dividing the IC<sub>50</sub> values in the cells transduced with Ad-LacZ vector by those in Ad-COX-2 AS-transduced cells.

### D. Determination of MDR1 expression by RT-PCR

Forty-eight or seventy-two hours after transduction of MCF-7/DOX cells with the indicated virus, total RNA was extracted from cells with RNA STAT- 60 kit (Tel-Test Inc, Friendswood, Tex) and quantified by UV absorbance spectroscopy. Total RNA (2  $\mu$ g) was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif) in a final volume of 50  $\mu$ L. The reaction mixture contained 1 $\times$  reverse transcriptase buffer (500 mM each deoxynucleotide triphosphate mixture [dNTP], 3 mM MgCl<sub>2</sub>, 75 mM KCl, 50 mM TRIS-HCl [pH 8.3]; 10 units RNase inhibitor; 10 mM dithiothreitol; 500  $\mu$ M per dNTP; 50 units reverse transcriptase; and 1.5 mM random hexamers. The thermal cycling parameters for reverse transcription reactions were as follows: 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. In a total volume of 50  $\mu$ L, the MDR1 and GAPDH were co-amplified. The reaction mixture contained the following ingredients: 2 $\times$  Ready Mix REDTaq PCR reaction mix, MDR1 forward primer (10 pmol/ $\mu$ L) [5'-ATA TCA GCA GCC CAC ATC AT-3'], MDR1 reverse primer (10 pmol/ $\mu$ L) [5-GAA GCA CTG GGA TGT CCG GT-3'], GAPDH forward primer (10 pmol/ $\mu$ L) [5'-GCC AAA AGG GTC ATC ATC TC-3], GAPDH reverse primer (10 pmol/ $\mu$ L) [5'-GTA GAG GCA GGG ATG ATG TTC-3']. The amplification cycles were as follows: initial denaturation step at 94°C for 3 minutes, then 33 cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5 minutes, followed by 72°C for 15 minutes. The PCR products were electrophoresed on 1.5% agarose gel, and band intensities were quantified using Alpha Imager Imaging System (Alpha Innotech Corporation, San Leandro, Calif), and the ratio of the intensity from MDR1 to GAPDH from each lane was graphed and analyzed.

### E. Western blotting

The cells were harvested 72 hours after transduction with either of the indicated viruses for protein analysis. The cells were washed in PBS, lysed in radioimmunoprecipitation (RIPA) lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% sodium dodecylsulfate (SDS), 1% nonidet-P40, 0.5% deoxycholate) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and protease inhibitors (phenylmethyl sulfonyl fluoride, leupeptin, and aprotinin) at the ratio of 100 ML buffer for 3  $\times$  10<sup>6</sup> cells. After 30 minutes on ice with some vortexing, the lysates were centrifuged at 13,000  $\times$  g for 20 minutes at 4°C. Protein concentration was determined with bicinchoninic acid protein assay kit (Pierce Technology Co, Rockford, Ill) according to the manufacturer's protocol. Equal amounts of proteins (20 Mg) were mixed with SDS reducing buffer. Protein samples were separated on acrylamide SDS-PAGE then transferred onto polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were

blocked with 5% nonfat milk in phosphate buffered saline-Tween (PBS-T) for 1 hour and then incubated overnight at 4°C with the corresponding primary antibodies. After three washes in 0.1% PBS-T, immunoreactive proteins were detected by using horseradish peroxidase-conjugated secondary antibodies (1:5000; Promega Corp, Madison, Wis). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL plus, Amersham Pharmacia Biotech). The intensity of each protein band was determined using a scanning densitometer (Epson 4870, Epson America, Long Beach, Calif).  $\beta$ -actin was used as internal control for normalization of the protein concentrations and loading precision.

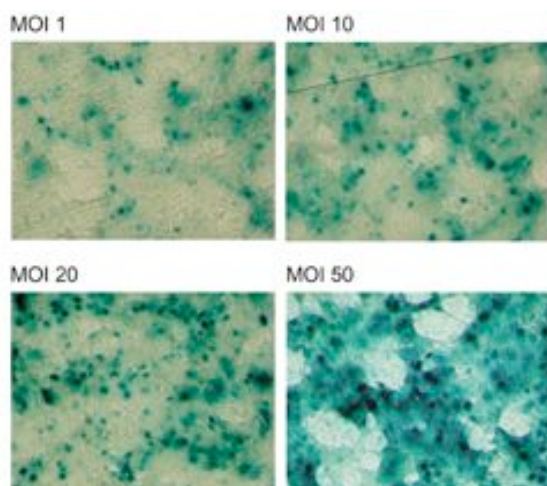
### F. Statistical analysis

All assays were performed in triplicate. The results were expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was determined by Student's *t* test. Significance was reached when *P* < 0.05.

## III. Results

### A. Adenovirus vector transduces MCF-7/DOX cells with higher efficiency compared with the parental MCF-7 cells

We first wanted to assess the ability of replication-incompetent adenovirus to transduce MCF-7/DOX cells versus the doxorubicin-sensitive parental MCF-7 cells. The transduction efficiency of the adenoviral vectors in MCF-7/DOX cells was tested using adenovirus LacZ (Ad-LacZ) vector. The adenovirus vector efficiently transduced MCF-7/DOX cells (**Figure 1**), and the transduction efficiency was proportional to the multiplicity of infections (MOI) used. The optimal transduction efficiency was obtained at an MOI of 100 plaque-forming units [PFU]/cell. At MOIs of 1, 10, 20, 50, and 100 PFU/cell, the percentages of  $\beta$ -galactosidase positive cells were 7  $\pm$  1.16, 17  $\pm$  3, 30  $\pm$  5.3, 85  $\pm$  1.5, and 95  $\pm$  1.5, respectively. On the other hand, Ad-lacZ vector transduced the parental cells (doxorubicin-sensitive MCF-7 cells) less efficiently compared with MCF-7/DOX cells. At similar MOIs (1, 10, 20, 50, and 100 PFU/cell), the percentages of  $\beta$ -galactosidase positive MCF-7 cells were 4  $\pm$  1.2, 11  $\pm$  1.8, 19  $\pm$  2.6, 60  $\pm$  3.8, and 85  $\pm$  2.7, respectively (**Table 1**).



**Figure 1.** Adenovirus-infected MCF-7/DOX cells. The transduction efficiency was MOI-dependent and about 85% of the cells were transduced at an MOI of 50 PFU/cell.

**Table 1.** Percentage of  $\beta$ -Galactosidase Positive Cells After Transducing MCF-7/DOX Cells or the Parental MCF-7 Cells With Different MOIs of Adenovirus-expressing  $\beta$ -Galactosidase.

Multiplicity of Infection (PFU/Cell)	Transduced Cells	
	MCF-7/DOX (%)	Parental MCF-7 Cells (%)
1	7 $\pm$ 1.16	4 $\pm$ 1.2
10	17 $\pm$ 3	11 $\pm$ 1.8
20	30 $\pm$ 5.3	19 $\pm$ 2.6
50	85 $\pm$ 1.5	60 $\pm$ 3.8
100	95 $\pm$ 1.5	85 $\pm$ 2.7

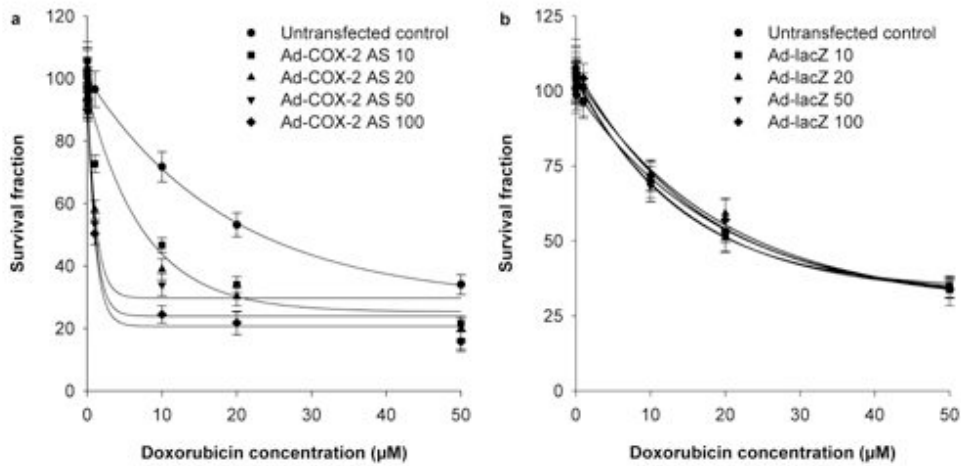
### B. Ad-COX-2 AS sensitizes MCF-7/DOX cells to doxorubicin

Based on the mounting evidence suggesting that overexpression of COX-2 in tumor cells confers resistance to chemotherapeutic agents, we have evaluated whether Ad-COX-2 AS has the therapeutic potential to modulate drug resistance in the doxorubicin-resistant breast cancer cell line MCF-7/DOX. Our data show that Ad-COX-2 AS sensitizes MCF-7/DOX cells to the cytotoxic effects of doxorubicin. Sensitivity to doxorubicin was assessed by a cytotoxicity assay, which compared the  $IC_{50}$  values in MCF-7/DOX cells transduced with Ad-COX-2 AS with  $IC_{50}$  values in cells transduced with the same MOI as Ad-LacZ. Transduction with Ad-COX-2 AS (MOI: 10, 20, 50, and 100 PFU/cell) lowered the  $IC_{50}$  of doxorubicin in MCF-7/DOX cells by 2.7-, 16-, 20- and 20-fold, respectively, compared with cells transduced with a similar MOI of Ad-LacZ (**Figure 2A**). However, Ad-LacZ did not influence the sensitivity of MCF-7/DOX cells to doxorubicin (**Figure 2B**). These data clearly indicated that Ad-COX-2 AS reversed the doxorubicin-resistant phenotype of MCF-7/DOX cells.

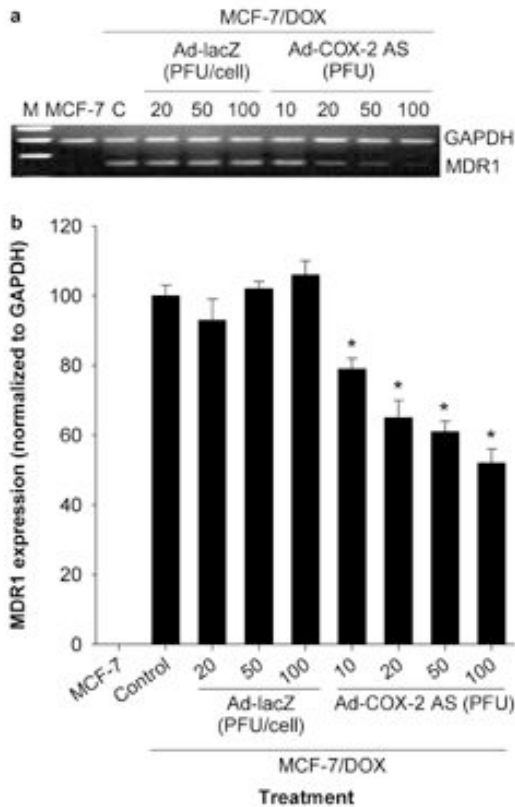
### C. Ad-COX-2 AS inhibits MDR1 gene expression

To determine if the transduction of MCF-7/DOX cells with Ad-COX-2 AS affects the expression of the MDR1 gene, and consequently the sensitivity to doxorubicin, the expression of MDR1 mRNA was quantified by reverse transcription polymerase chain reaction (RT-PCR) in MCF-7/DOX cells transduced with the different MOI of Ad-LacZ or Ad-COX 2 AS. Transduction of MCF-7/DOX with Ad-LacZ (20–100 PFU/cell) did not alter the MDR1 expression (**Figure 3A**). However, Ad-COX-2 AS (MOI 10, 20, 50, and 100 PFU/cell) significantly downregulated MDR1 gene expression by 21%, 35%, 39%, and 48%, respectively, compared with the Ad-LacZ-transduced cells or untransduced control cells (**Figure 3A, 3B**).

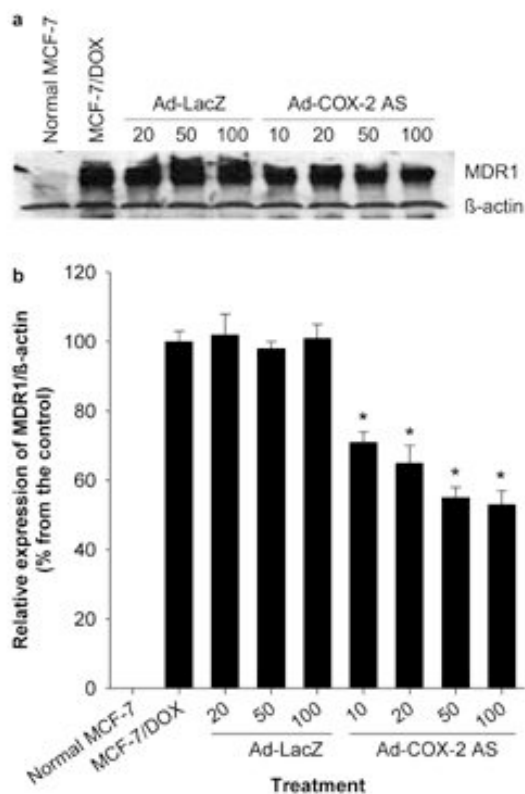
To confirm the effect of Ad-COX 2 AS on the MDR1 gene expression, Western blot analysis was used to evaluate the MDR1 protein level. Consistent with its effect on the mRNA level, Ad-COX 2 AS reduced the MDR1 protein expression in MCF-7/DOX cells (**Figure 4A**). Quantitatively, transduction of MCF-7/DOX cells with Ad-COX-2 AS at MOI of 10, 20, 50, or 100 PFU/cell significantly reduced MDR1 protein expression levels by 29%, 35%, 45%, and 47%, respectively, compared with untransduced or Ad-LacZ-transduced cells (**Figure 4B**).



**Figure 2.** Effect of different MOIs of (A) Ad-COX-2 AS or (B) Ad-LacZ on the sensitivity of MCF-7/DOX cells to doxorubicin. MCF-7/DOX cells were transduced with Ad-COX-2 AS or Ad-LacZ at different MOIs. Twenty-four hours after transduction, the cells were treated with different concentrations of doxorubicin for 72 hours. Then, the cell proliferation was determined and the IC<sub>50</sub> was calculated. Values represent the mean ± SEM from three similar experiments.



**Figure 3.** (A) Effect of Ad-LacZ or Ad-COX-2 AS on MDR1 mRNA expression in MCF-7/DOX cells. MCF-7/DOX cells were transduced with the different MOI of Ad-LacZ or Ad-COX-2 AS. Forty-eight hours later, RNA was extracted and the MDR1 mRNA was amplified using RT-PCR. GAPDH was co-amplified and used as internal control. The PCR products were electrophoresed on 1.5% agarose gel. Lane 1: DNA marker; Lane 2: normal MCF-7 cells; Lane 3: control MCF-7/Dox cells; Lane 4–6: MCF-7/DOX transduced with Ad-LacZ (20, 50, and 100 PFU/cell); Lane 7–10: MCF-7/DOX transduced with Ad-COX-2 AS (10, 20, 50, and 100 PFU/cell). (B) The ratio of the band intensity of MDR1 to GAPDH from the RT-PCR. Values are expressed as mean ± SD (\* $P < 0.01$ ). This figure shows the results of quantifying the density of PCR products by Alpha Imager Imaging System. \*Significantly different from untransduced control cells and cells transduced with Ad-LacZ ( $P < 0.05$ ). Data are presented as the mean ± SEM of three independent experiments.



**Figure 4.** (A) Effect of Ad-COX-2 AS on MDR1 protein expression in MCF-7/DOX cells. The cells were transduced with the different MOI of Ad-LacZ or Ad-COX-2 AS. Seventy-two hours later, cell lysates were prepared and an equal amount (50 Mg) of protein was separated by 8% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with monoclonal antiMDR1 antibody or antibody against  $\beta$ -actin. Lane 1: normal MCF-7 cells; Lane 2: untransduced MCF-7/DOX cells; Lane 3–5: MCF-7/DOX cells Ad-LacZ (10, 20, 50, and 100 PFU/cell); Lane 6–9, MCF-7/DOX transduced with Ad-COX-2 AS (10, 20, 50, and 100 PFU/cell). (B) Results of quantifying the density of protein bands by Alpha Imager Imaging System. \*Significantly different ( $P < 0.05$ ) compared with control MCF-7/DOX cells and cells transduced with Ad-LacZ using two-sided distribution (Student's  $t$  test). Results are the representative of three similar experiments (mean  $\pm$  SE).

#### D. Ad-COX-2 AS downregulates cyclin D1 expression in MCF-7/DOX cells

Compelling evidence suggests a role for COX-2 in the growth of cancer cells via induction of the expression of cell cycle regulating genes. Therefore, we investigated the effect of Ad-COX-2 AS on cyclin D1 expression in MCF-7/DOX cells. As expected, Ad-COX-2 AS downregulates the expression of cyclin D1 in MCF-7/DOX cells in an MOI-dependent fashion (Figure 5A). Transduction with Ad-COX-2 AS at MOI of 10, 20, 50, and 100 PFU/cells downregulates cyclin D1 expression in MCF-7/DOX cells by 22%, 35%, 42%, and 43%, respectively, compared with cells transduced with Ad-LacZ or untransduced control cells (Figure 5B).

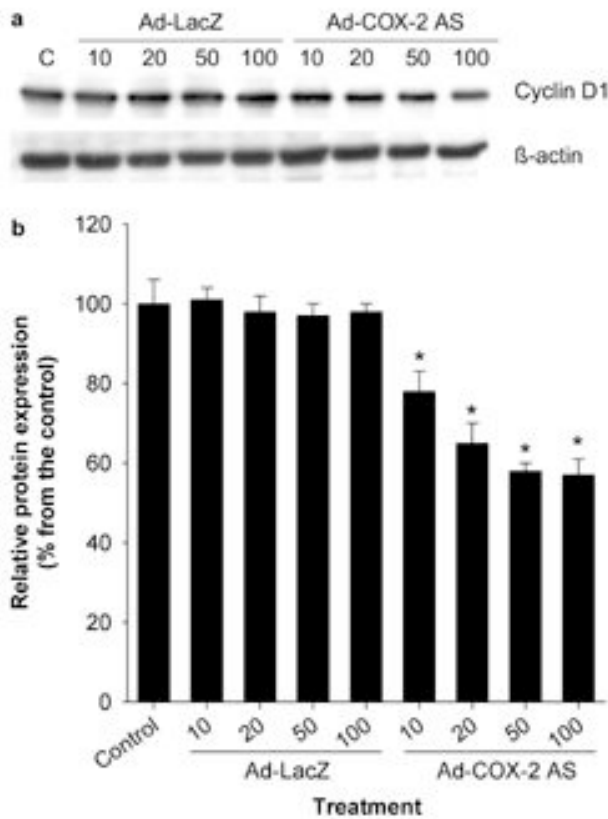
#### E. Effect of Ad-COX-2 AS on Bcl-2 and Bax expression in MCF-7/DOX cells

Selective inhibitor of COX-2 has been reported to inhibit growth and induce apoptosis in several cancer cell lines (Sanborn and Blanke, 2005; Pyrko et al, 2006). Thus, we examined expression of the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax in MCF-7/DOX cells after transduction with Ad-COX-2 AS. Western blotting analysis demonstrated that Ad-COX-2 AS significantly reduced the expression of Bcl-2 protein (Figure 6A). Compared with untransduced control cells and Ad-LacZ

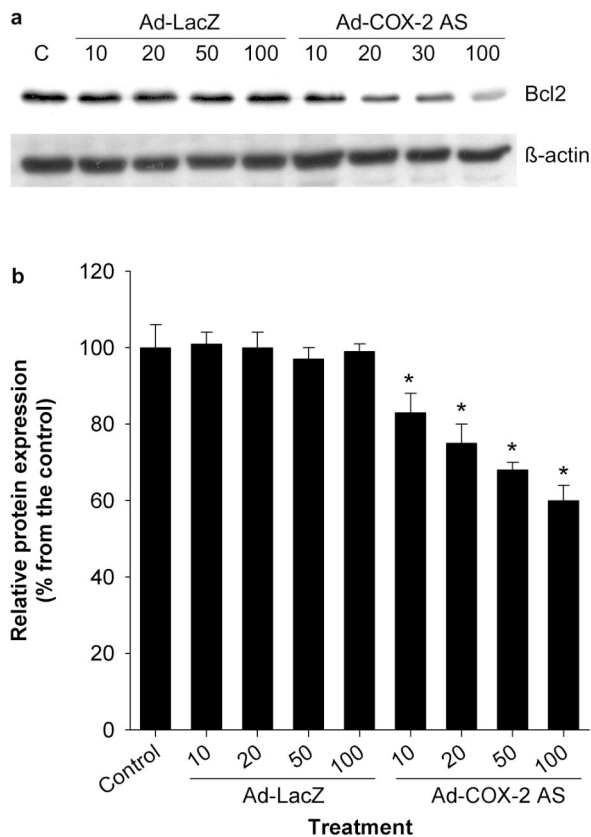
transduced cells, Ad-COX-2 AS at MOIs of 10, 20, 50, and 100 PFU/cells decreased the Bcl-2 expression by 17%, 25%, 32%, and 40%, respectively (Figure 6B). On the other hand, Ad-COX-2 AS had a minimal effect on Bax expression (Figure 7A, 7B).

## IV. Discussion

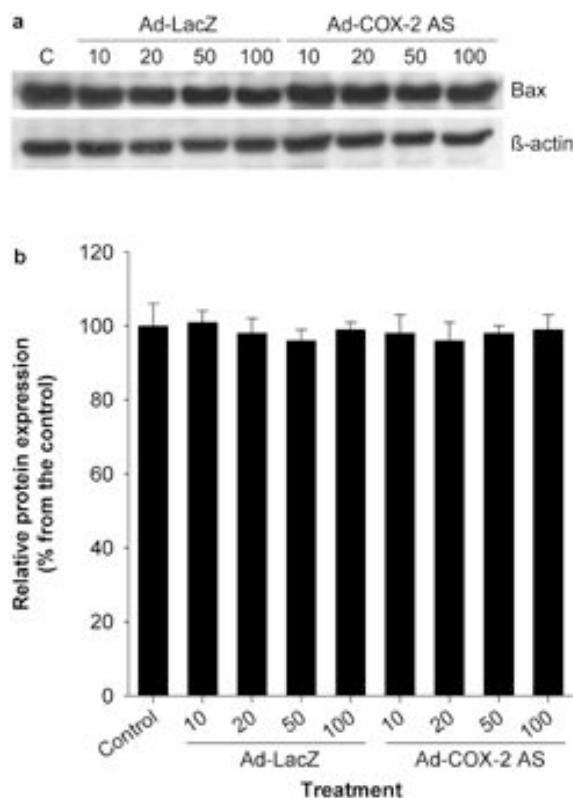
COX-2 overexpression is reported in many malignancies, including breast cancer (Ranger et al, 2004; Schmitz et al, 2006; Singh et al, 2006). Elevated tumor prostaglandin  $E_2$ , a major COX-2 metabolite, has been implicated in angiogenesis, tumor growth, invasion, reduced apoptosis, and resistance to anticancer drugs (Raspollini et al, 2005; Surowiak et al, 2005; Zatelli et al, 2005; Krysan et al, 2006). A functional role of COX-2 in tumor development and progression has been demonstrated by both overexpression (Liu et al, 2001) and disruption (Chulada et al, 2000) of the COX-2 gene as well as application of drugs blocking either COX-1/-2 or COX-2 alone (Wang and DuBois, 2004; Harris et al, 2005; Harris et al, 2006). It has been reported that COX-2 upregulates MDR1/P-gp expression, which confers the resistance to anticancer drugs (Ratnasinghe et al, 2001; Patel et al, 2002; Ziemann et al, 2002; Sorokin 2004; Surowiak et al, 2005). In addition, COX-2 expression is



**Figure 5.** Expression of cyclin D1 protein as measured with Western blot in MCF-7/DOX cells transduced with the different MOI of Ad-LacZ or Ad-COX-2 AS. MCF-7/DOX cells were transduced with Ad-LacZ or Ad-COX-2 AS. Seventy-two hours after transduction, cell lysates were prepared and equal amounts were separated by 12% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with monoclonal anti-cyclin D1 antibody or antibody against  $\beta$ -actin. (A) Results of developing the x-ray film. Lane 1: untransduced MCF-7/DOX cells; Lane 2–5: MCF-7/DOX cells Ad-LacZ (10, 20, 50, and 100 PFU/cell); Lane 6–9: MCF-7/DOX transduced with Ad-COX-2 AS (10, 20, 50, and 100 PFU/cell). (B) Results of quantifying protein expression by Alpha Imager Imaging System. \*Significantly different ( $P < 0.05$ ) compared with control MCF-7/Dox cells and cells transduced with Ad-LacZ using two-sided distribution (Student's  $t$  test). Results are the mean  $\pm$  SEM for three different experiments.



**Figure 6.** Expression of Bcl-2 protein as measured with Western blot in MCF-7/DOX cells transduced with the different MOI of Ad-LacZ or Ad-COX-2 AS. MCF-7/DOX cells were transduced with Ad-LacZ or Ad-COX-2 AS. Seventy-two hours after transduction, cell lysates were prepared and equal amounts were separated by 12% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with monoclonal anti-Bcl-2 antibody or antibody against  $\beta$ -actin. (A) Results of developing the x-ray film. Lane 1: untransduced MCF-7/DOX cells; Lane 2–5: MCF-7/DOX cells Ad-LacZ (10, 20, 50, and 100 PFU/cell); and Lane 6–9: MCF-7/DOX transduced with Ad-COX-2 AS (10, 20, 50, and 100 PFU/cell). (B) Results of quantifying protein expression by Alpha Imager Imaging System. \*Significantly different ( $P < 0.05$ ) compared with control MCF-7/Dox cells and cells transduced with Ad-LacZ using two-sided distribution (Student's  $t$  test). Results are the mean  $\pm$  SEM for three different experiments.



**Figure 7.** Expression of Bax protein as measured with Western blot in MCF-7/DOX cells transduced with the different MOI of Ad-LacZ or Ad-COX-2 AS. MCF-7/DOX cells were transduced with Ad-LacZ or Ad-COX-2 AS. Seventy-two hours after transduction, cell lysates were prepared and equal amounts were separated by 12% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with monoclonal antiBax antibody or antibody against  $\beta$ -actin. (A) Results of developing the x-ray film. Lane 1: untransduced MCF-7/DOX cells; Lane 2–5: MCF-7/DOX cells Ad-LacZ (10, 20, 50, and 100 PFU/cell); Lane 6–9: MCF-7/DOX transduced with Ad-COX-2 AS (10, 20, 50, and 100 PFU/cell). (B) Results of quantifying protein expression by Alpha Imager Imaging System. \*Significantly different ( $P < 0.05$ ) compared with control MCF-7/DOX cells and cells transduced with Ad-LacZ using two-sided distribution (Student's  $t$  test). Results are expressed as mean  $\pm$  SEM for three different experiments.

associated with inhibition of the apoptotic pathway in tumor cells (Tsuji and DuBois 1995; Meric et al, 2006), and it induces cell proliferation by upregulation of cyclin D1 (Wu et al, 2004; Patel et al, 2005; Zhang et al, 2006). Therefore, COX-2 inhibitors have been used as chemopreventive and therapeutic agents in cancer treatment (Chow et al, 2005; Harris et al, 2006; Mazhar et al, 2006). Unfortunately, currently available orally administered COX-2 inhibitors are not optimal compounds, and their use is associated with serious cardiovascular effects (Becerra et al, 2003; Dogne et al, 2006; Meric et al, 2006). In fact, the US Food and Drug Administration has recently ordered rofecoxib to be withdrawn from the American market due to increased cardiovascular and thrombotic risks (Dogne et al, 2006; Schnitzer 2006). Others systemic COX-2 inhibitors have also been voluntarily withdrawn. Thus, development of strategies that target COX-2 selectively into tumor cells with minimal systemic toxicity will be a welcomed addition to cancer treatment.

The effects of COX-2 in gene expression are generally mediated by formation of cAMP, the downstream effector of COX-2. Generally, genes regulated by COX-2 harbor cAMP response element (CRE) in their promoter regions. MDR1, Cyclin D1, and Bcl-2 genes have been reported to be up-regulated by cAMP, an effect mediated through cAMP response element (CRE) in their promoter regions (Chang et al, 2006; Xiang et al, 2006; Ziemann et al, 2006). Thus, abrogation of COX-2 expression using the Ad-COX-2 AS can cause a decrease in the cellular level of cAMP with subsequent down

regulation of MDR1, Cyclin D1, and Bcl-2 genes expression. Although the mechanism by which decrease cyclin D1 gene expression affect the sensitivity of tumor cells to doxorubicin is not known, it has been reported that the expression of genes that control the cell cycle are of critical importance in determining the sensitivity of cells and tumors to drugs (chemosensitivity) and radiation. It has been consistently reported tumor cell lines expressing higher of Cyclin D1 demonstrated resistance to cytotoxic drugs compared with cells expressing lower levels (Hochhauser et al, 1996; Henriksson et al, 2006).

A gene therapy strategy could be the answer to this dilemma, since it provides effective localized delivery of a particular gene product while avoiding undesirable systemic side effects. In this study, we test the feasibility of using Ad-COX-2 AS as a gene therapy approach to selectively shut down COX-2 enzyme expression in tumor cells. The impact of transducing the MCF-7/DOX cells with Ad-COX-2 AS upon the expression of MDR1, Bcl-2, Bax, and cyclin D1 protein expression as well as the sensitivity to doxorubicin cytotoxicity was assessed. Our results demonstrated that the adenovirus vector transduces MCF-7/DOX cells more efficiently compared with doxorubicin-sensitive parental cells. This finding is consistent with several earlier reports suggesting that adenovirus vector preferentially transduces anticancer-resistant tumor cells more efficiently compared with the parental cell line (Shirakawa et al, 2001; Ambriovic-Ristov et al, 2004; Holm et al, 2004).

Our results also indicated that transduction of MCF-7/DOX cells with Ad-COX-2 AS resulted in a dramatic



improvement in the sensitivity of these cells to the cytotoxic effect of doxorubicin. This reversal of the chemoresistance phenotypes is associated with downregulation of MDR1 gene expression. These observations are consistent with the well-documented effect of COX-2 inhibitors on sensitizing the tumor cells to chemotherapeutic agents and the regulation of MDR1 gene expression (Sorokin 2004; Puhlmann et al, 2005; Zatelli et al, 2005). Furthermore, Ad-COX-2 AS downregulated the expression of the antiapoptotic gene Bcl-2 and the cell cycle promoting gene cyclin D1. Similar effects have been observed with the conventional COX-2 inhibitors (Takada et al, 2004; Arellanes-Robledo et al, 2006).

When the gene therapy era launched, it became evident that cancer would be an appropriate target for this developing field. Most importantly, the application of the gene therapy approach, which can be adapted to transcriptional targeting into a particular tissue or cell type, or certain cancer cells, represents a novel therapy that is distinct from traditional COX-2 inhibitors. The substantial advances in the adenovirus vector design raised expectations for this adenovirus-mediated gene therapy approach to be advantageous to other vectors (Al Hendy and Salama, 2006; Glasgow et al, 2006; Rein et al, 2006). Vector genomes can be manipulated to express therapeutic transgenes specifically into tumor cells utilizing transductional and transcriptional targeting strategies. Contrary to the conventional COX-2 inhibitors, the deployment of the Ad-COX-2 AS approach can avoid the undesirable systemic side effects on various organs. In conclusion, this “proof-of-concept” study suggests that gene therapy modalities using adenovirus vector expressing Ad-COX-2 AS are a promising treatment modality for breast cancer, especially in patients who have had unsuccessful traditional chemotherapeutic treatment.

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