Enhancement of Doxorubicin Cytotoxicity on Human Esophageal Squamous Cell Carcinoma Cells by Indomethacin and 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (SC236) via Inhibiting P-Glycoprotein Activity⒮

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ABSTRACT

Doxorubicin is a chemotherapeutic drug widely used for the treatment of advanced esophageal squamous cell carcinoma. However, its efficacy is usually limited by the development of multidrug resistance (MDR), which has been linked to the up-regulation of P-glycoprotein (P-gp) in cancer cells. Conventional nonsteroidal anti-inflammatory drugs and cyclooxygenase 2 (COX-2)-selective inhibitors have been demonstrated to overcome MDR in some cancer cells. Here we sought to elucidate the effect of COX inhibitors on doxorubicin-induced cytotoxicity in relation to P-gp function in human esophageal squamous cell carcinoma cells. Among the five tested COX inhibitors [indomethacin, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzenesulfonamide (SC236), 5-(4-chlorophenyl)-1-(4-methoxy-phenyl)-3-trifluorom-ethylpyrazole (SC560), nimesulide, and *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398)], all of which substantially suppressed prostaglandin E_2 (PGE_2) production to a similar extent, only the nonselective COX inhibitor indomethacin and the COX-2-selective inhibitor SC236 enhanced cytotoxic effects of doxorubicin on HKESC-1 and HKESC-2 cells. Moreover, these effects could not be reversed by the addition of PGE_2. Knockdown of COX-2 by small interference RNA also failed to mimic the enhancing effects of indomethacin or SC236, implicating that their action is COX- and PGE_2-independent. To this end, we observed that indomethacin and SC236 directly functioned as noncompetitive inhibitors of P-gp, which were manifested as a reduction of P-gp ATPase activity. Collectively, these findings suggest that the direct inhibitory effects of indomethacin and SC236 on P-gp may contribute to their ability to increase the intracellular retention of doxorubicin and thus enhance its cytotoxicity. The combination of indomethacin or SC236 with doxorubicin may have significant potential clinical application, especially in the circumvention of P-gp-mediated MDR in cancer cells.

Esophageal cancer, a highly aggressive neoplasm with dismal prognosis, is the sixth leading cause of cancer-related death in the world (Tew et al., 2005). Although a continuous increase in the incidence of adenocarcinoma of the esophagus has been reported, the most frequently diagnosed histological subtype is still the squamous cell carcinoma (Blot and McLaughlin, 1999; Stoner and Gupta, 2001). Several strategies, including preoperative chemotherapy, either alone or with radiotherapy, and chemoradiotherapy without surgery, have been used in attempts to improve the prognosis (Wobst et al., 1998; Stahl et al., 2005). Neoadjuvant chemotherapy using a regimen consisting of 5-fluorouracil, cisplatin, and doxorubicin (Adriamycin) has been reported to be effective in

ABBREVIATIONS: MDR, multidrug resistance; SC236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide; SC560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluorom-ethylpyrazole; NS398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; nimesulide, *N*-(4-nitro-2-phenoxyphenyl)methanesulfonamide; PGE_2, prostaglandin E_2; COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; siRNA, small interference RNA; P-gp, P-glycoprotein; MRP1, multidrug resistance protein 1; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline.

the treatment of advanced esophageal carcinoma (Naritaka et al., 2004; Kosugi et al., 2005; Akita et al., 2006; Yano et al., 2006; Shimakawa et al., 2008). Compared with the regimen consisting of 5-fluorouracil and cisplatin, 5-fluorouracil/cisplatin/doxorubicin exhibits much higher response rate and promising long-term outcomes for patients with highly advanced esophageal carcinoma (Kosugi et al., 2005).

The development of multidrug resistance (MDR) in cancer cells represents one of the major causes of failure in anticancer chemotherapy. A major contributor to this phenomenon is increased drug efflux mediated by P-glycoprotein (P-gp), a 170-kDa ATP-dependent membrane transporter that acts as a drug efflux pump decreasing intracellular drug accumulation and therefore reducing intracellular drug efficacy (Gillet et al., 2007; Mimeault et al., 2008). Accordingly, inhibitory agents that target P-gp function are of therapeutic interest. From a pharmacological perspective, P-gp function may be inhibited as a result of competitive or noncompetitive antagonism. Competitive inhibitors act as substrates to compete with cytotoxic agent for transportation by P-gp, limiting the efflux of the cytotoxic agent and increasing its intracellular concentration. Conversely, noncompetitive inhibitors bind with high affinity to P-gp but are not themselves substrates, which prevent ATP hydrolysis and transport of cytotoxic agent out of the cell, resulting in an increased intracellular concentration (Thomas and Coley, 2003). Doxorubicin is a well known P-gp substrate. Its cytostatic efficacy is thus limited by P-gp activity (Gillet et al., 2007; Mimeault et al., 2008). It is therefore not surprising that P-gp inhibitors have been demonstrated to reverse resistance to doxorubicin in a variety of cancer cells in vitro and in vivo (Sato et al., 1995; Nakanishi et al., 1997; David-Beabes et al., 2000).

The chemotherapeutic and chemoprophylactic potentials of nonsteroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase 2 (COX-2)-selective inhibitors, have been suggested in a variety of cancers, including esophageal squamous cell carcinoma (Zimmermann et al., 1999; Corley et al., 2003; Stoner et al., 2005). Different mechanisms have been demonstrated to contribute to their antitumor action, such as the suppression of cell proliferation (Goldberg et al., 1996), the induction of apoptosis (Shao et al., 2000; Hashitani et al., 2003), and the inhibition of angiogenesis (Jones et al., 1999; Masferrer et al., 2000). COX inhibitors also enhance the action of cytostatic drugs. In this regard, it has been suggested that COX inhibitors overcome the MDR of tumors to chemotherapeutic drugs like doxorubicin by inhibiting P-gp in some human cancers such as breast cancer, colorectal cancer, medullary thyroid carcinoma, and acute myeloid leukemic cells (Awara et al., 2004; Puhlmann et al., 2005; Zatelli et al., 2005; Zatelli et al., 2007; Zrieki et al., 2008). However, their action on esophageal cancer has not been reported. Furthermore, the mechanism for both NSAIDs and COX-2-selective inhibitors on P-gp activity is still obscure. In the present study, we addressed these issues in vitro by studying the cytotoxic effects of NSAIDs, COX-1, and COX-2 selective inhibitors in combination with doxorubicin on human esophageal squamous cell carcinoma cells.

Materials and Methods

Chemicals and Drugs. SC236 (specific COX-2 inhibitor) was purchased from Pfizer (New York, NY). SC560 (specific COX-1 in-

hibitor) and antibody to COX-2 were obtained from Cayman Chemical (Ann Arbor, MI). NS398 (specific COX-2 inhibitor) was from Calbiochem (La Jolla, CA). COX-2 small interference (siRNA) and control siRNA were from QIAGEN GmbH (Hilden, Germany). Antibodies against P-gp, multidrug resistance protein 1 (MRP1), and NF-κB p65 subunit were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lipofectamine 2000 reagent and fluorescent-labeled RNA duplex were from Invitrogen (Carlsbad, CA). Reagents for electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA). All of the other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Cell Culture. Human esophageal squamous cell carcinoma cell lines, HKESC-1 and HKESC-2, were kindly provided by Prof. G. Srivastava (Department of Pathology, The University of Hong Kong, Hong Kong, China). HKESC-1 and HKESC-2 were established from moderately differentiated human esophageal squamous cell carcinoma (Hu et al., 2000, 2002). These two cell lines were maintained in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide Assay. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium (MTT) assay, which depends on the ability of viable cells to reduce the MTT to a colored formazan product. In brief, cells were seeded in 96-well microculture plates overnight for attachment and then incubated for 24 h with different concentrations of doxorubicin in the presence or absence of NSAID, specific COX-1 inhibitor, and specific COX-2 inhibitors. In the next step, MTT was added to each well, and the cells were further incubated for 3 h. The colored formazan product was determined photometrically at 570 nm in a multiwell plate reader (Bio-Rad Laboratories).

Confocal Microscopy Analysis. The confocal fluorescent microscopy was used to compare the intracellular accumulation and retention of doxorubicin. Cells were seeded in Petri dish and incubated overnight for attachment, after which doxorubicin was added. Cells were incubated in medium containing doxorubicin for 6 h. Subsequently, the culture medium was removed, and cells were washed three times with PBS. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and washed three times with PBS. Intracellular doxorubicin fluorescent signals were visualized by using a Nikon ECLIPSE TE2000-E confocal microscope (Nikon, Tokyo, Japan). Doxorubicin fluorescence was excited with an argon laser at 488 nm, and the emission was collected through a 530-nm long-pass filter.

Flow Cytometry Analysis. Flow cytometry was used to quantify the intracellular accumulation and retention of doxorubicin. Cells were washed twice in PBS, harvested after trypsin treatment, and washed twice again. Then the doxorubicin fluorescence was measured using Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA). Using the excitation with an argon laser at 488 nm, emission of 10,000 events per sample was detected on FL2 (575 nm).

Western Blot. Cell were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% α-cholate acid, 0.1% SDS, 2 mM EDTA, 1% Triton X-100, and 10% glycerol) containing protease and phosphatase inhibitors. After sonication for 30 s on ice and centrifugation for 15 min at 12,000g at 4°C, the supernatant was collected, and protein concentration was determined by assay kit (Bio-Rad Laboratories). Equal amounts of protein (100 μg/lane) were resolved with SDS-polyacrylamide gel electrophoresis and transferred to Hybond C nitrocellulose membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The membranes were probed with primary antibodies overnight at 4°C and incubated for 1 h with secondary peroxidase-conjugated antibody at room temperature. Chemiluminescent signals were then developed with Lumiglo reagent (Cell Signaling Technology Inc., Danvers, MA) and detected by the ChemiDoc XRS documentation system (Bio-Rad Laboratories).
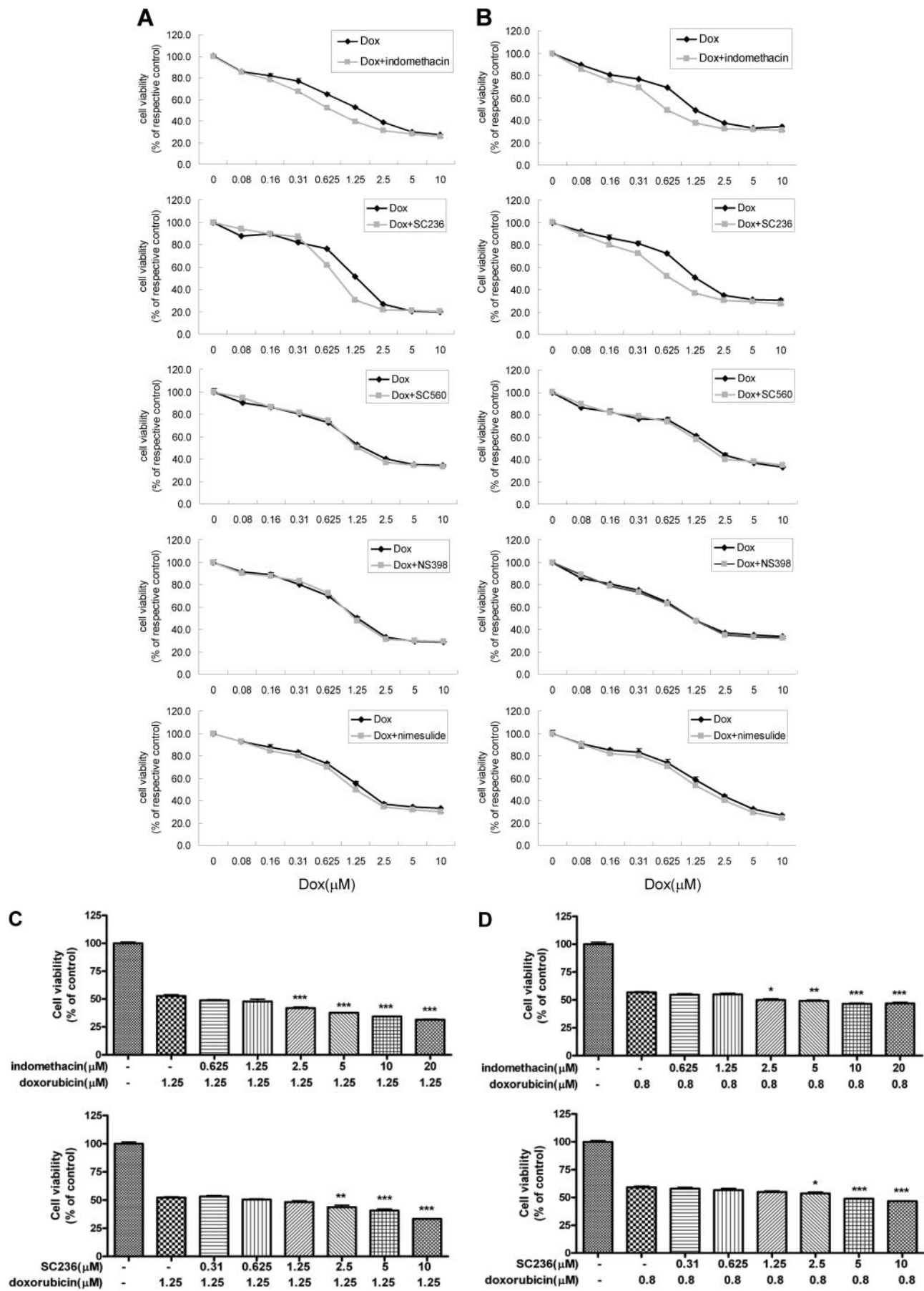
Nuclear and Cytosolic Extracts. Nuclear and cytosolic extracts were isolated by using the Nuclear Extraction Kit (Cayman) according to the manufacturer's recommendations. The extracted nuclear and cytosolic fractions were then subjected to Western blot analysis.

RNA Interference. The expression of COX-2 was lowered using predesigned target-specific siRNA molecules. COX-2 or control siRNA was transfected into cells at 40 to 60% confluence using Lipofectamine 2000 reagent according to the manufacturer's instructions. Nontargeting siRNA was used as control siRNA, which has no homology to any known mammalian gene.

PGE$_2$ Assay. The measurement of PGE$_2$ in the cell culture medium was carried out by using the Correlate-EIA Prostaglandin E$_2$ Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instruction. In brief, cells were plated in a 24-well microculture plates in the presence of 10% serum. At confluence, fresh medium containing 1% serum in the presence or in the absence of tested NSAID, specific COX-1 inhibitor, or selective COX-2 inhibitors was added. Cells were incubated in medium containing tested compounds for 24 h, after which supernatants were collected for PGE$_2$ measurement. The PGE$_2$ level was expressed as picogram per milliliter per microgram of protein.

P-gp ATPase Assay. Activity of P-gp ATPase in response to doxorubicin and COX inhibitors was determined by Pgp-Glo assay system (Promega, Madison, WI). By following the user protocol provided by the vender, the activity of P-gp ATPase was measured in the presence of verapamil (as a positive conference), doxorubicin, or tested COX inhibitors. The luminescence of the sample reflects the ATP level in the sample, which is negatively correlated with the activity of P-gp ATPase and was recorded using the Wallac Victor 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Monza, Italy). Test compound-treated activities are expressed as the percentage of basal activity. By comparing basal activity to test compound-treated activities, the compounds can be ranked as stimulating, inhibiting, or having no effect on basal P-gp ATPase activity.

Statistical Analysis. Results were expressed as mean ± S.E.M. Statistical analysis was performed with an analysis of variance followed by the Turkey's t test. P values less than 0.05 were considered statistically significant.

Results

Indomethacin and SC236 Enhanced Doxorubicin-Induced Cytotoxicity Both in HKESC-1 and HKESC-2 Cells. To study whether COX inhibitors could enhance cytotoxic action of doxorubicin, HKESC-1 cells (Fig. 1A) and HKESC-2 cells (Fig. 1B) were treated with doxorubicin (0–10 μM) in the absence or presence of the NSAID indomethacin (20 μM), COX-2-selective inhibitors SC236 (10 μM), NS398 (10 μM), and nimesulide (10 μM), or COX-1-selective inhibitor SC560 (20 μM). Doxorubicin decreased cell viability dose-dependently both in HKESC-1 and HKESC-2 cells with an EC$_{50}$ value of approximately 1.2 and 0.8 μM, respectively. Cotreatment of HKESC-1 cells with indomethacin or SC236, both of which alone had no effect on cell viability, decreased the EC$_{50}$ value from 1.2 to 0.4 and 0.6 μM, respectively. Likewise, indomethacin and SC236 also enhanced the cytotoxic action of doxorubicin in HKESC-2 cells, which decreased the EC$_{50}$ value from 0.8 to 0.3 and 0.4 μM, respectively. In contrast, COX-1-selective inhibitor SC560 and the

other two tested COX-2 selective inhibitors NS398 and nimesulide exerted no effect on the action of doxorubicin in both cell lines. To determine the minimal effective concentrations of indomethacin and SC236, HKESC-1 cells were incubated with various concentrations of indomethacin or SC236 in combination with doxorubicin at the concentration of 1.25 μM, which was around its EC$_{50}$ value for HKESC-1 cells. Both indomethacin and SC236 at the concentration as low as 2.5 μM significantly enhanced the cytotoxic action of doxorubicin (Fig. 1C). Likewise, these two COX-inhibitors using the same concentration significantly sensitized HKESC-2 cells to the cytotoxic action of doxorubicin (0.8 μM, the EC$_{50}$ for HKESC-2 cells) (Fig. 1D).

Indomethacin and SC236 Increased Intracellular Accumulation and Retention of Doxorubicin in HKESC-1 Cells. Doxorubicin is an autofluorescent compound, which enables the visualization of its presence by confocal microscopy. Figure 2A showed the fluorescence of intracellular accumulated doxorubicin in HKESC-1 cells after incubation for 6 h with 1.25 μM doxorubicin in the absence or presence of 20 μM indomethacin or 10 μM SC236. Fluorescent signals in cells cotreated with doxorubicin and indomethacin or SC236 were much stronger than those in cells treated with doxorubicin alone, indicating that both indomethacin and SC236 were able to increase the intracellular accumulation of doxorubicin. For the doses tested (1.25, 5, and 20 μM), the minimal concentration of indomethacin to significantly increase intracellular accumulation of doxorubicin was 5 μM (Fig. 2B). In parallel, different doses (0.625, 2.5, and 10 μM) of SC236 were examined. The minimal concentration of SC236 to significantly increase doxorubicin accumulation was 2.5 μM (Fig. 2C). In contrast, fluorescent signals in cells cotreated with doxorubicin and SC560 (20 μM), nimesulide (10 μM), or NS398 (10 μM) were similar to those in cells treated with doxorubicin alone (Supplementary Fig. 1A).

In relation to intracellular retention of doxorubicin, Fig. 2D showed HKESC-1 cells that were incubated for 6 h with 1.25 μM doxorubicin followed by incubation for another 6 h in normal growth medium or in medium containing 20 μM indomethacin or 10 μM SC236. Results showed that only a small amount of previously accumulated doxorubicin left in cells after withdrawal of doxorubicin for 6 h. In contrast, cells treated with indomethacin or SC236 contained much more doxorubicin than those that were incubated in normal growth medium after doxorubicin withdrawal. These findings indicated that both indomethacin and SC236 increased the intracellular retention of doxorubicin. Again, for the doses tested (1.25, 5, and 20 μM), the minimal concentration of indomethacin to significantly increase intracellular retention of doxorubicin was 5 μM (Fig. 2E). In parallel, for the doses tested (0.625, 2.5, and 10 μM), the minimal concentration of SC236 to significantly affect doxorubicin retention was 2.5 μM (Fig. 2F). Unlike indomethacin and SC236, SC560, nimesulide, or NS398 failed to augment doxorubicin retention in cells (Supplementary Fig. 1B).

Fig. 1. Effects of COX inhibitors on doxorubicin-induced cytotoxicity in HKESC-1 cells and HKESC-2 cells. HKESC-1 cells (A) or HKESC-2 cells (B) were treated for 24 h with doxorubicin (0–10 μM) alone or in combination with indomethacin (20 μM), SC236 (10 μM), SC560 (20 μM), NS398 (10 μM), or nimesulide (10 μM). Cell viability was then determined by MTT assay. Data are presented as the mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. C, HKESC-1 cells were incubated with 1.25 μM doxorubicin under treatment of various concentrations of indomethacin or SC236 for 24 h. D, HKESC-2 cells were incubated with 0.8 μM doxorubicin under treatment of various concentrations of indomethacin or SC236 for 24 h. Cell viability was then determined by MTT assay. Data are presented as the mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. ∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$ compared with doxorubicin-treated group.

PGE₂ Failed to Reverse the Enhancing Effect of Indomethacin or SC236 on Cytotoxicity of Doxorubicin in HKESC-1 Cells. Because the enhancing effect of indomethacin or SC236 on the cytotoxicity of doxorubicin may be due to COX-2 inhibition, we therefore determined whether the COX-2 end product PGE_2 could reverse the observed effects. The basal PGE_2 level released by HKESC-1 cells was 332 pg/ml, or approximately 10^{-3} μM (data not shown). Moreover, it has been reported that PGE_2 at the concentration of 3 μg/ml (8.5 μM) neutralized the enhancing effect of the COX-2 selective inhibitor meloxicam on the cytotoxic action of doxorubicin in acute myeloid leukemia HL-60 cells (Puhlmann et al., 2005). Therefore, PGE_2 at concentrations ranging from 10^{-3} to 10 μM were used in our study. As shown in Fig. 3, A and B, PGE_2 at all tested concentrations failed to reverse the enhancing effect of indomethacin or SC236 on the cytotoxicity of doxorubicin.

The involvement of PGE_2 in the enhancing effect of indomethacin or SC236 was further excluded by the findings that SC560, NS398, and nimesulide suppressed PGE_2 release from HKESC-1 cells to a similar extent to that of indomethacin or SC236 (Fig. 3C); however, unlike indomethacin or SC236, these three COX inhibitors failed to enhance the cytotoxic action of doxorubicin on cancer cells. In addition, indomethacin at three tested concentrations (1.25, 5, and 20 μM) significantly decreased PGE_2 release to a similar extent in which there was no significant difference among these concentrations (Fig. 3D). However, indomethacin at concen-
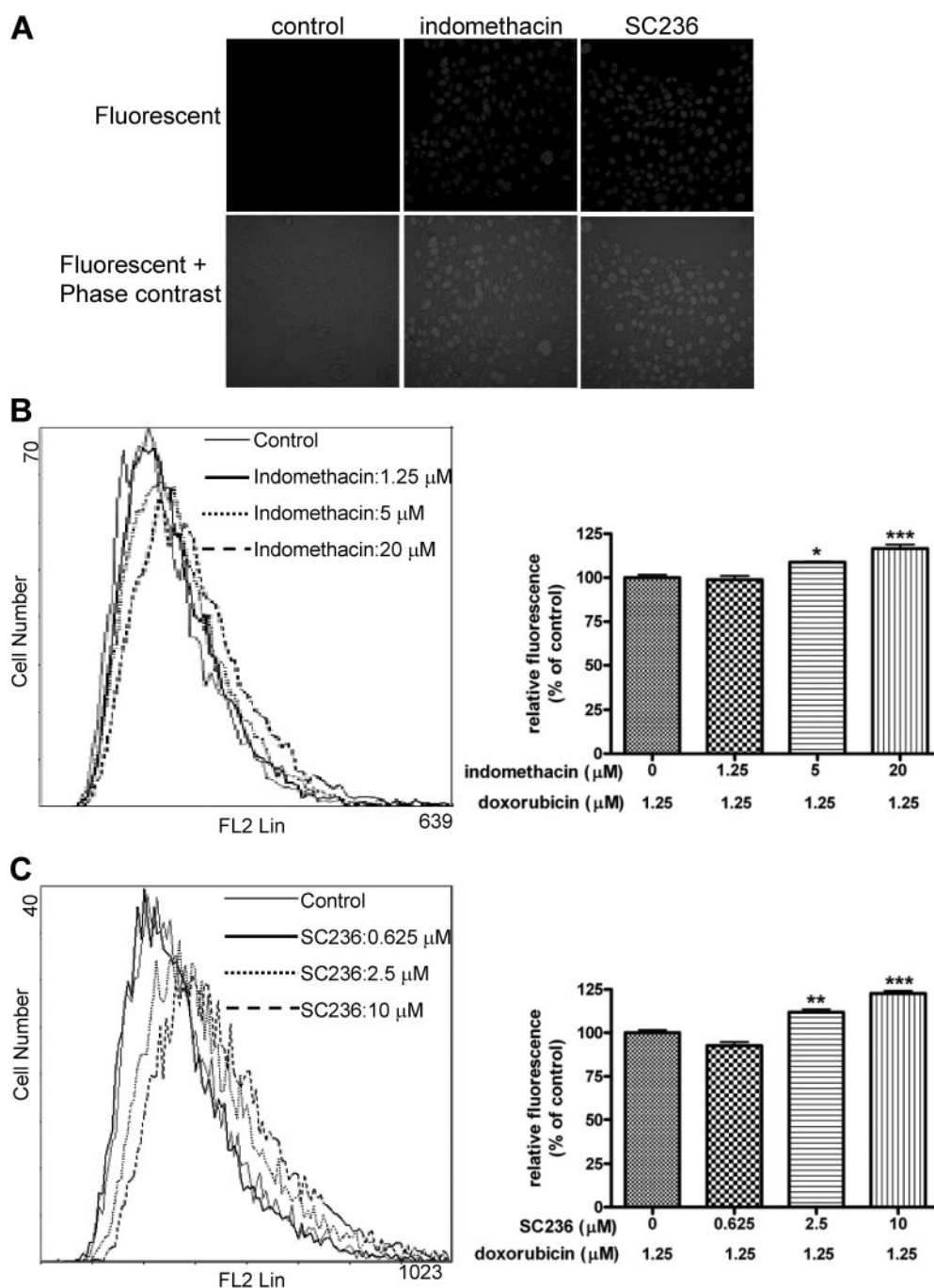

Fig. 2. Effects of indomethacin and SC236 on the intracellular accumulation and retention of doxorubicin in HKESC-1 cells. A, shows fluorescent microscopic pictures of HKESC-1 cells that were incubated for 6 h in medium containing 1.25 μM doxorubicin alone or in combination with 20 μM indomethacin or 10 μM SC236. Magnification, 400×. These results are representative of three independent experiments. Histograms of the fluorescence intensity of doxorubicin in HKESC-1 cells under treatment of doxorubicin (1.25 μM) alone or in combination with various concentrations of indomethacin (B) or SC236 (C). Data are presented as mean± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. ∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$ compared with doxorubicin-treated group.

trations of 5 and 20 μM, not 1.25 μM, enhanced the cytotoxicity of doxorubicin. In parallel, SC236 at three tested concentrations (0.625, 2.5, and 10 μM) significantly reduced PGE$_2$ release to a similar extent (Fig. 3E). However, SC236 only at the concentrations of 2.5 and 10 μM, not 0.625 μM, enhanced the cytotoxic action of doxorubicin. These findings further suggest that PGE$_2$ is not involved in the enhancing effect of indomethacin and SC236.

Down-Regulation of COX-2 Expression Was Unable to Enhance Doxorubicin-Induced Cytotoxicity in HKESC-1 Cells. Because only SC236 among the three tested COX-2 inhibitors showed enhancing effect on doxorubicin-induced cytotoxicity, the direct role of COX-2 in this action was further investigated by RNA interference experiments. The results showed that the siRNA transfection efficacy was more than 90% by using fluorescent-labeled RNA (Fig. 4A). Moreover, the efficacy of COX-2 depletion by specific siRNA was further verified by Western blot analysis (Fig. 4B). The effect of COX-2 siRNA on PGE$_2$ production was also determined. In this regard, down-regulation of COX-2 expression by COX-2 siRNA significantly reduced PGE$_2$ production in HKESC-1 cells (Fig. 4C). In relation to the role of COX-2 in cytotoxic action of doxorubicin, Fig. 4D showed that down-regulation of COX-2 was unable to enhance doxorubicin-induced cytotoxicity, indicating that inhibition of COX-2 is not required for the enhancing effect of indomethacin or SC236.
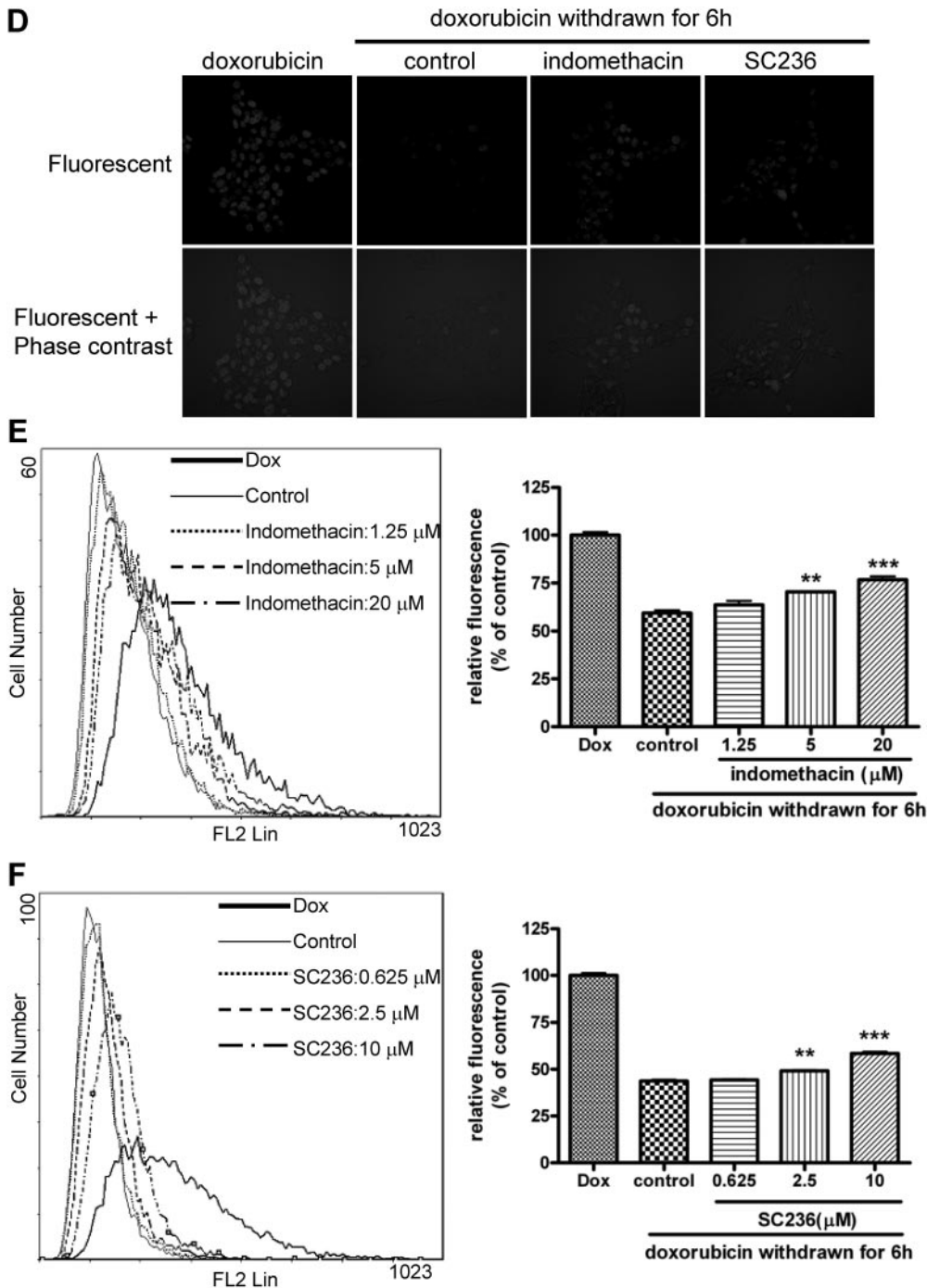

Fig. 2. *Continued.* D, fluorescent microscopic picture of HKESC-1 cells treated with 1.25 μM doxorubicin for 6 h followed by 6-h incubation in normal growth medium or medium containing 20 μM indomethacin or 10 μM SC236. Magnification, 400×. These results are representative of three independent experiments. Histograms of the fluorescence intensity of doxorubicin in HKESC-1 cells under treatment of doxorubicin (1.25 μM) for 6 h followed by 6-h incubation in normal growth medium or medium containing various concentrations of indomethacin (E) or SC236 (F). Data are presented as mean ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. ∗∗, $p < 0.01$ and ∗∗∗, $p < 0.001$ compared with cells treated with doxorubicin for 6 h followed by 6-h incubation in normal growth medium.

Indomethacin and SC236 Showed No Inhibitory Effect on NF-κB. Because COX-2 inhibitors have been demonstrated to increase intracellular doxorubicin accumulation and subsequently enhance its cytotoxicity in human breast tumor cells through inhibition of NF-κB activity, as indicated by the reduced nuclear translocation of the p65 subunit (van Wijngaarden et al., 2007), we examined the effects of indomethacin and SC236 on NF-κB activity in HKESC-1 cells. In an inactive state, NF-κB is sequestered in the cytoplasm as a heterodimer consisting of p50, p65, and IκBα subunits. In response to an activation signal, the IκBα subunit becomes phosphorylated, ubiquitinated, and ultimately degraded through the proteasomal pathway. This process exposes the

Fig. 3. Effects of supplementation of PGE$_2$ on enhancement of doxorubicin cytotoxicity by indomethacin and SC236 in HKESC-1 cells. Cells were treated with or without 1.25 μM doxorubicin in the absence or presence of PGE$_2$ (10^{-3} to 10 μM) and 20 μM indomethacin (A) or 10 μM SC236 (B) alone or in combination for 24 h before determination of cell viability by MTT assay. Data are presented as the means ± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. Cells were treated with indicated COX inhibitors (20 μM indomethacin, 10 μM SC236, 20 μM SC560, 10 μM nimesulide, or 10 μM NS398) (C), various concentrations of indomethacin (D), or SC236 (E) for 24 h. Supernatants were then collected for PGE$_2$ measurement. The PGE$_2$ level was expressed as picogram per milliliter per microgram of protein. Data are presented as mean± S.E.M. ($n = 3$) of a representative experiment performed in triplicate. ∗∗∗, $p < 0.001$ compared with control group.

nuclear localization signals on the p50-p65 heterodimer, facilitating its nuclear entry, binding to specific sequence in DNA and activating transcription of target genes (Dorai and Aggarwal, 2004). In the present study, results showed that neither indomethacin nor SC236 altered the cytosolic or nuclear expressions of p65 subunit (Fig. 5A). Furthermore, combination of doxorubicin with indomethacin or SC236 also showed no influence on the cytosolic and nuclear p65 staining (Fig. 5B). These findings indicated that indomethacin and SC236 did not affect on NF-κB activity.

Indomethacin and SC236 Inhibited P-gp ATPase Activity. The results presented so far indicated that indomethacin or SC236 enhanced cytotoxic effect of doxorubicin is not mediated through the inhibition of COX-2 and subsequent PGE$_2$ production and inhibition of NF-κB. Although both indomethacin and SC236 increased intracellular accumulation and retention of doxorubicin that is a substrate for a membrane drug efflux pump P-gp, whether this phenomenon was related to P-gp activity via COX-independent manner has not yet been determined. As shown in Fig. 6A, doxorubicin, indomethacin, and SC236 alone or in combination did not alter the expression of P-gp. However, doxorubicin obviously increased P-gp ATPase activity to a comparable extent with that of the positive control verapamil, which functions as a P-gp competitive inhibitor (Fig. 6B). Unlike doxorubicin, indomethacin or SC236 significantly decreased P-gp ATPase activity, indicating that both of these COX inhibitors functioned as P-gp noncompetitive inhibitors (Fig. 6C). Moreover, other tested COX inhibitors (SC560, nimesulide, and NS398) showed no effects on P-gp ATPase activity (Supplementary Fig. 2). In addition, indomethacin at concentrations of 5 and 10 μM and SC236 at concentrations of 2.5 and 10 μM significantly reduced doxorubicin-induced P-gp ATPase activity (Fig. 6, D and E), which agreed with the effects of indomethacin and SC236 on intracellular accumulation and retention of doxorubicin. These findings suggest that indomethacin or SC236 enhanced cytotoxic action of doxorubicin through direct inhibitory actions on P-gp activity in cancer cells.

Discussion

Here we show that the nonselective COX inhibitor indomethacin and the COX-2-selective inhibitor SC236 enhanced the cytotoxicity of doxorubicin in human esophageal squamous cell carcinoma cells, HKESC-1 and HKESC-2. Indomethacin and SC236 also substantially increased the intracellular accumulation and retention of doxorubicin. The enhancement effects were independent of COX and the subsequent PGE$_2$ production, as well as of NF-κB. In an attempt to explain the enhancement of doxorubicin cytotoxicity caused by indomethacin and SC236, the effect of these two compounds on the function of P-gp was investigated. To this end, both indomethacin and SC236 acted as noncompetitive inhibitors of P-gp ATPase, which retarded the efflux of doxorubicin and thus augmented its cytotoxicity.

A growing body of evidence has demonstrated that NSAIDs and COX-2-selective inhibitors enhance the cytotoxic action of certain chemotherapeutic drugs in a variety of cancer cells (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999; Awara et al., 2004; O'Connor et al., 2004; Puhlmann et al., 2005; Zatelli et al., 2005, 2007; Zrieki et al., 2008). In agreement with these findings, our results show that the NSAID
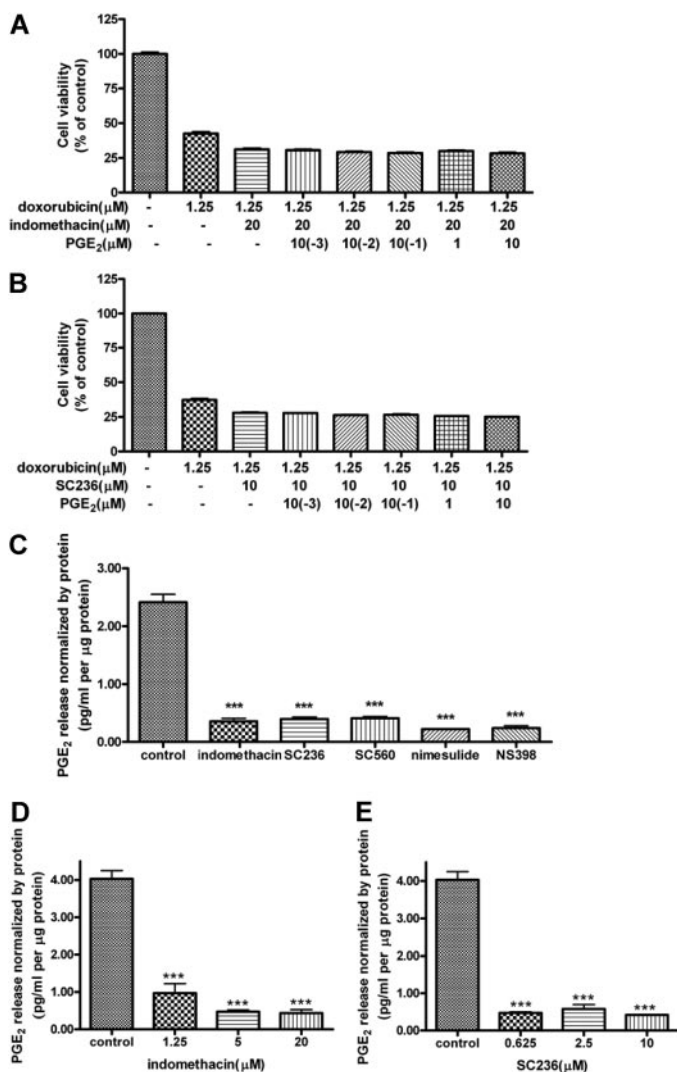
indomethacin and the COX-2-selective inhibitor SC236 sensitize human esophageal squamous cell carcinoma cells (HKESC-1 and HKESC-2) to the cytotoxic action of doxorubicin. Similar enhancing effect of indomethacin and SC236 was also observed in a human gastric adenocarcinoma cell line TMK1 (Supplementary Fig. 3). Although both compounds are COX inhibitors, we present evidence that their enhancing effect on the cytotoxicity of doxorubicin is COX-independent. First of all, unlike SC236, COX-2-selective inhibitors NS398 and nimesulide and COX-1-selective inhibitor SC560 showed no influence on cytotoxic action of doxorubicin, although all of these tested COX inhibitors suppressed PGE_2 production to a similar extent. Moreover, exogenous supplementation of COX product PGE_2 failed to reverse the enhancing effect of indomethacin or SC236. In addition to pharmacological approach, siRNA-mediated knockdown of COX-2 also showed no effect on the cytotoxic action of doxorubicin, which provides the direct evidence for a COX-2-independent mechanism.

Apart from the inhibition of COX enzymes, it has been suggested that additional mechanisms are involved in the actions of NSAIDs and COX-2-selective inhibitors. One possible mechanism is that these inhibitors repress NF-κB activity, which is increased in response to chemotherapeutic agents like doxorubicin (Nakanishi and Toi, 2005). More noteworthy is that a recent study demonstrated COX-2-selective inhibitors increased the intracellular accumulation of doxorubicin and enhanced doxorubicin-induced cytotoxicity in human breast cancer cells via inhibition of doxorubicin-induced NF-κB activation (van Wijngaarden et al., 2007). Unlike these findings, our studies show that NF-κB activity is not increased in response to doxorubicin treatment. Furthermore, neither indomethacin nor SC236 exerted inhibitory effect on NF-κB activity in the absence or in the presence of doxorubicin. These results suggest that NF-κB is not in-

volved in the enhancing effect of indomethacin or SC236 on doxorubicin-induced cytotoxicity. This discrepancy between the findings of our study and others may be due to the intrinsic difference among the cell lines used.

The efficacy of chemotherapeutic drugs is greatly compromised by emergence of the MDR, which is regarded as a major obstacle to effective cancer chemotherapy. Such a resistance pattern is mainly mediated by ATP-binding cassette transporters via ATP-dependent drug efflux. Among these ATP-binding cassette transporters, P-gp and MRP1 are two major transporters that function as pumps to extrude doxorubicin from cancer cells (Gillet et al., 2007; Mimeault et al., 2008). It has been suggested that COX inhibitors may sensitize cancer cells to chemotherapeutic drugs like doxorubicin via inhibiting P-gp (Awara et al., 2004; Puhlmann et al., 2005; Zatelli et al., 2005, 2007; Zrieki et al., 2008) or MRP1 (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999; O'Connor et al., 2004). Although COX inhibitors like indomethacin has been reported to reverse MRP-mediated efflux of doxorubicin via inhibition of MRP1 pumping system (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999), the involvement of MRP1 in augmentation of doxorubicin toxicity by indomethacin or SC236 is excluded in our study based on the findings that MRP1 protein is undetectable in HKESC-1 and HKESC-2 cells (data not shown). In contrast, P-gp expression is expressed in esophageal squamous cell carcinoma cell lines. However, its expression is quite stable in response to doxorubicin in the absence or in the presence of indomethacin or SC236. We therefore directly measured the activity of P-gp. In this respect, doxorubicin functions as a substrate for P-gp, which is manifested as increased P-gp ATPase activity. Conversely, both indomethacin and SC236 act as noncompetitive inhibitors for P-gp, decreasing the basal P-gp ATPase activity and doxorubicin-induced P-gp ATPase activity and thereby preventing the transport of
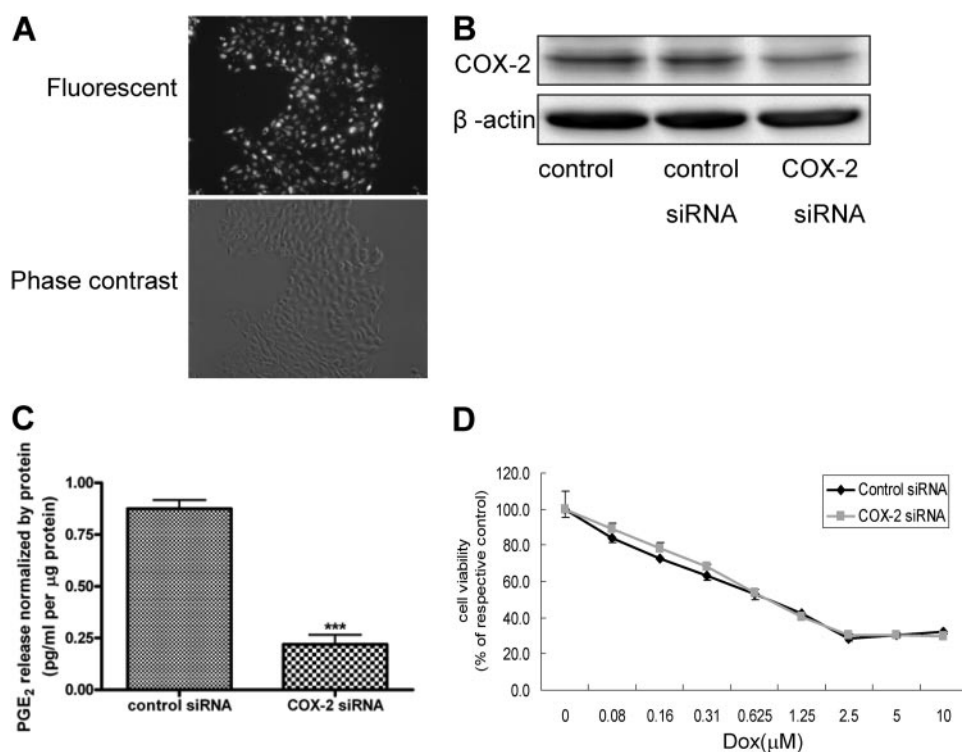

Fig. 4. Effects of siRNA-mediated knockdown of COX-2 on doxorubicin-induced cytotoxicity in HKESC-1 cells. A, the transfection efficiency of siRNA was determined by transfection of fluorescent-labeled RNA duplex in HKESC-1 cells. These results are representative of three independent experiments. B, the efficacy of COX-2 depletion by COX-2 siRNA was verified by Western blot analysis. Nontargeting siRNA was used as control siRNA. β-Actin was used to evaluate protein loading. These results are representative of three independent experiments. C, after transfection with the control siRNA or COX-2 siRNA, cells were incubated in growth medium for 24 h. Supernatants were then collected for PGE_2 measurement. The PGE_2 level was expressed as picogram per milliliter per microgram of protein. Data are presented as the means ± S.E.M. (n = 3) of a representative experiment performed in triplicate. ***, $p < 0.001$ compared with control siRNA-transfected group. D, after transfection with the control siRNA or COX-2 siRNA, cells were treated with doxorubicin at indicated concentrations for 24 h before MTT assay. Data are presented as the means ± S.E.M. (n = 3) of a representative experiment performed in triplicate.

doxorubicin out of cells. These findings suggest a direct inhibitory action of indomethacin or SC236 on P-gp function, which may contribute to the increased intracellular doxorubicin accumulation and retention as well as the subsequent enhancement of cytotoxicity on esophageal cancer cells.

Throughout the last two decades, much effort has been made to identify agents that are able to inhibit P-gp as a way to reverse MDR. A variety of agents with different chemical

Fig. 5. Western blot detection of NF-κB subunit p65 in nuclear and cytosolic extracts from HKESC-1 cells. A, cells were incubated for 6 h in the absence or the presence of 1.25 μM doxorubicin, 20 μM indomethacin, or 10 μM SC236. B, cells were incubated for 6 h with 1.25 μM doxorubicin alone or in combination with 20 μM indomethacin or 10 μM SC236. These results are representative of three independent experiments.

structures that modulate the function of P-gp have been identified. These agents can be categorized pharmacologically into competitive inhibitors and noncompetitive inhibitors. Early agents like verapamil belong to competitive inhibitors and work by competing with the chemotherapeutic drugs for efflux by the P-gp pump. One of the drawbacks of this group of compounds is that high serum concentrations are necessary to produce adequate intracellular concentrations of the chemotherapeutic drugs. Thus, they typically reverse MDR at concentrations that result in unacceptable toxicity. In contrast, noncompetitive inhibitors act via noncompetitive binding to P-gp pump and thereby overcome the limitations of competitive inhibitors, which are more promising for the therapeutic application (Thomas and Coley, 2003). It is noteworthy that both indomethacin and SC236 are most likely to function as noncompetitive inhibitors for P-gp, which manifested by the inhibition of P-gp ATPase activity. The minimal effective concentration (2.5 μM) of indomethacin used in the present experiments is almost three times lower than that in the plasma of the patients treated with indomethacin in the clinical trial (Helleberg, 1981). With respect to SC236, it has been reported that SC236 at the plasma concentration of 5 μg/ml (12.5 μM), which is five times higher than the minimal effective concentration (2.5 μM) in our study, was used in an orthotopic xenograft mouse model (Lee et al., 2006). Although both indomethacin and SC236 at their effective concentrations in the present study showed no cytotoxic action on the tested cells in vitro, given the concerns regarding the safety of COX inhibitor, further safety study on the combination of these COX inhibitors with doxorubicin in vivo is warranted.
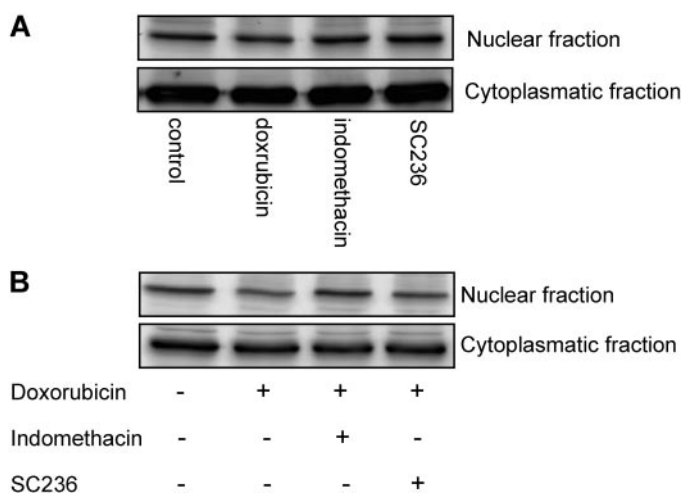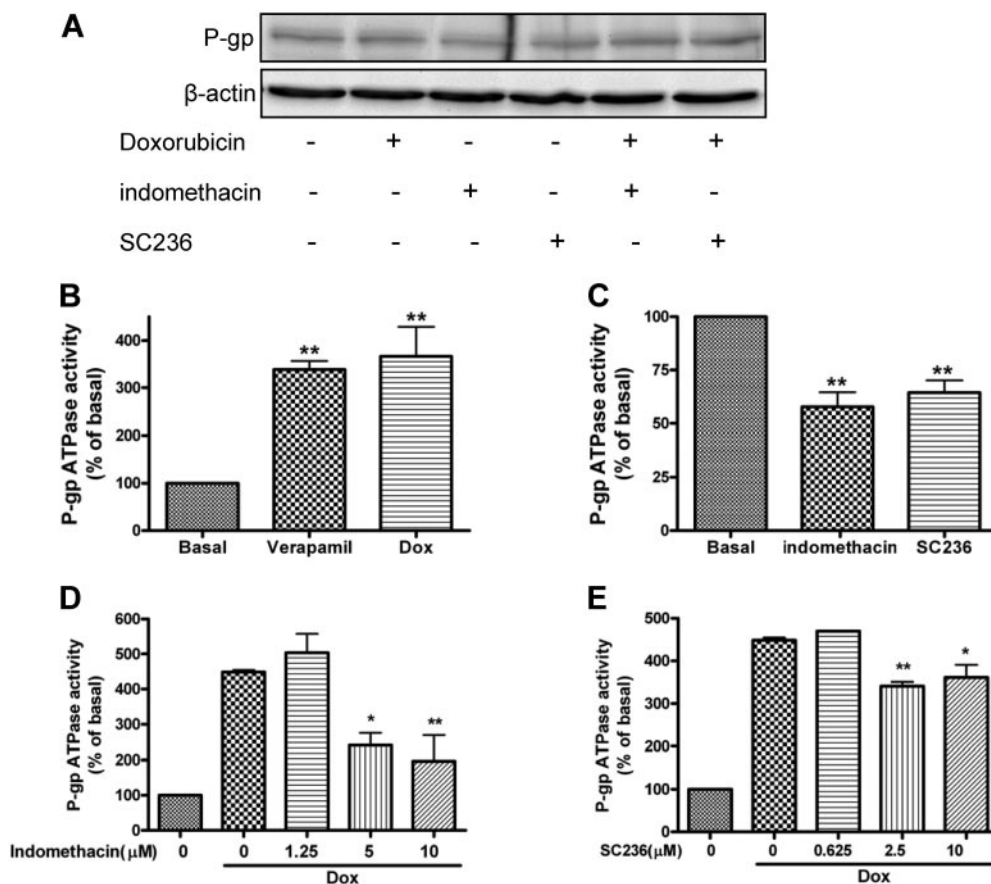

Fig. 6. Effects of indomethacin and SC236 on P-gp ATPase activity. A, cells were incubated for 6 h with 1.25 μM doxorubicin alone or in combination with 20 μM indomethacin or 10 μM SC236. Cells were then collected for the determination of P-gp expression by Western blot analysis. β-Actin was used to evaluate protein loading. These results are representative of three independent experiments. P-gp ATPase activity in response to the treatment of 10 μM doxorubicin (B) or 20 μM indomethacin and 10 μM SC236 (C). Results are expressed as the means ± S.E.M. (n = 3) of three independent experiments. **, $p < 0.01$ compared with basal level of P-gp ATPase activity. D, effects of indomethacin (1.25, 5, and 20 μM) on 10 μM doxorubicin-induced P-gp ATPase activity. E, effects of SC236 (0.625, 2.5, and 10 μM) on 10 μM doxorubicin-induced P-gp ATPase activity. Results are expressed as the means ± S.E.M. (n = 3) of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with doxorubicin-treated group.

In conclusion, our studies show a new mechanism by which indomethacin and SC236 exert an enhancing effect on the cytotoxic effect of doxorubicin via direct inhibition of P-gp ATPase activity in human esophageal squamous cell carcinoma cells. Based on these findings, the combination of indomethacin or SC236 with doxorubicin may have potential clinical applications, especially in the circumvention of P-gp-mediated multidrug resistance in cancer cells.

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