

# Celecoxib Antagonizes the Cytotoxicity of Cisplatin in Human Esophageal Squamous Cell Carcinoma Cells by Reducing Intracellular Cisplatin Accumulation

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## ABSTRACT

High cyclooxygenase 2 (COX-2) expression has been reported to be clinically associated with reduced cisplatin-based therapy efficacy in esophageal cancer. However, the benefit of including COX-2-selective inhibitors in therapeutic regimens remains uncertain. Thus, we sought to determine the effects of COX inhibitors on the cytotoxicity of cisplatin and to further explore the mechanism involved in human esophageal squamous cell carcinoma cells. Among the four tested COX inhibitors [celecoxib, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (SC-236), nimesulide, and indomethacin], all of which substantially suppressed prostaglandin E<sub>2</sub> production to a similar extent; only the COX-2-selective inhibitors celecoxib and SC-236 antagonized cisplatin-induced cytotoxicity and apoptosis in both cisplatin-resistant cells and their wild-type counterparts. Knockdown of COX-2 by small interference RNA failed to mimic the antagonizing effects of celecoxib and SC-236, implying that

their action is COX-2-independent. Further mechanistic analysis revealed that the antagonizing effect of celecoxib and SC-236 on cytotoxic action of cisplatin was associated with decreased whole-cell cisplatin accumulation and DNA platination. Reduced influx, accompanied by the reduction of protein level of copper transporter 1, accounts for decreased intracellular cisplatin accumulation. In addition, combined treatment did not elicit greater antitumor activity than cisplatin or celecoxib monotherapy in vivo in an esophageal cancer xenograft model. Collectively, these data demonstrate that celecoxib antagonizes the cytotoxicity of cisplatin by decreasing intracellular cisplatin and DNA platination. The combination treatment also shows no beneficial effect compared with cisplatin or celecoxib monotherapy in vivo. Therefore, current clinical trials with celecoxib in combination with cisplatin should be approached with caution.

## Introduction

Esophageal cancer, a highly aggressive neoplasm with dismal prognosis, is the ninth most common malignancy and sixth leading cause of cancer-related death in the world (Siersema, 2008). Upon histological examination, esophageal cancer can be categorized into two major subtypes: adenocarcinoma and squamous cell carcinoma. Although the incidence

of the former has increased in the United States and Europe over the past 3 decades, esophageal squamous cell carcinoma is still the dominant histological subtype worldwide (Ekman et al., 2008). For patients with locoregional esophageal cancer, surgical resection is the best treatment option for cure. However, nearly 50% of patients diagnosed with esophageal cancer present with metastasis, and chemotherapy is the mainstay of palliation in this setting (Ilson, 2008).

Cisplatin is one of the most effective and widely used chemotherapeutics for the treatment of various human malignant solid and metastatic tumors, including esophageal squamous cell carcinoma (Toshimitsu et al., 2004). Unfortunately, the development of cisplatin resistance in cancer cells is a major obstacle for successful treatment outcomes. Resis-

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**ABBREVIATIONS:** COX-2, cyclooxygenase 2; NSAID, nonsteroidal anti-inflammatory drug; SC-236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide; MS, mass spectrometry; siRNA, small interference RNA; ELISA, enzyme-linked immunosorbent assay; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PBS, phosphate-buffered saline; ICP, inductively coupled plasma; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PARP, poly(ADP-ribose) polymerase; CTR1, copper transporter 1.

tance to cisplatin can be intrinsic or acquired (Martin et al., 2008). Cellular resistance to cisplatin is multifactorial. Several mechanisms of cisplatin resistance have been postulated, including diminished accumulation of cisplatin, increased detoxification of drugs by the thiols, such as glutathione and metallothionein, and improved repair of and tolerance to nuclear DNA lesions leading to a concomitant reduction in apoptosis (Hall et al., 2008). Accordingly, agents that can circumvent drug resistance or enhance cytotoxic action of cisplatin are of therapeutic interest, and among these are found the cyclooxygenase 2 (COX-2)-selective inhibitors.

The chemotherapeutic and chemoprophylactic potential of nonsteroidal anti-inflammatory drugs (NSAIDs), including COX-2-selective inhibitors, has been demonstrated in various cancers, including human esophageal squamous cell carcinoma (Zimmermann et al., 1999; Corley et al., 2003; Stoner et al., 2005). It has been suggested that different mechanisms contribute to their antitumor action, including the attenuation of cell proliferation (Goldberg et al., 1996), inhibition of angiogenesis (Jones et al., 1999; Masferrer et al., 2000), suppression of invasion and metastasis (Takahashi et al., 2002), and induction of apoptosis (Shao et al., 2000; Hashitani et al., 2003). In addition to their antitumor activity as a monotherapy, it has also been suggested that COX-2-selective inhibitors can, in some cases, enhance the action of chemotherapeutic agents when incorporated into treatment regimens. For example, COX-2-selective inhibitors potentiated cisplatin cytotoxicity in a variety of human cancer cells in vitro, such as tongue squamous cell carcinoma, vulvar cancer, bladder cancer, and gastric cancer (Sugiura et al., 2003; Mizutani et al., 2004; Kim et al., 2009; Li et al., 2010). Several lines of opposing evidence, however, have shown that COX-2 inhibitors antagonized or exerted no effect on cytotoxicity of cisplatin in various cancer cells. In the case of ovarian cancer and colon cancer cells, COX-2-selective inhibitors have been reported to antagonize the cytotoxic action of cisplatin (Bijman et al., 2008). In another study, a panel of three human non-small-cell lung cancer cell lines was employed to test the effects of COX-2-selective inhibitors on cisplatin cytotoxicity. The results showed that COX-2-selective inhibitors exerted no effect on cisplatin cytotoxicity in two of the three tested cell lines, whereas they enhanced cisplatin cytotoxicity in the other cell line (Kilic et al., 2009). It is difficult to reconcile with intrinsic difference among tumors of different origins. To date, most investigators reporting on the role of COX-2-selective inhibitors in cisplatin cytotoxicity have based their findings on the quantitative degree of change of cisplatin cytotoxicity without further mechanistic explorations.

Several phase II trials have been reported on the COX-2-selective inhibitor celecoxib coadministered with cisplatin-based chemoradiotherapy for locally advanced esophageal cancer (Tew et al., 2005; Dawson et al., 2007). However, the results from different trials are conflicting regarding the therapeutic benefit of including celecoxib in the treatment regimens. Otherwise, to the best of our knowledge, the role of celecoxib in cisplatin cytotoxicity thus far has not been elucidated in human esophageal squamous cell carcinoma by any ex vivo experiment or xenograft model. Therefore, in the present study, we addressed these issues, both in vitro and in vivo, by studying the cytotoxic effects of a nonselective NSAID or COX-2-selective inhibitors in combination with

cisplatin on human esophageal squamous cell carcinoma cells. Moreover, we explored the mechanisms underlying the interaction between COX-2-selective inhibitors and cisplatin. We anticipate that this study will bring new insight into the combined use of COX-2-selective inhibitors and cisplatin in malignant solid tumors.

## Materials and Methods

**Reagents and Chemicals.** 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (SC-236) was purchased from Pfizer (New York, NY). Celecoxib was kindly provided by Dr. Q. H. Zhu (School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China). Using MS and <sup>1</sup>H NMR, the identity of celecoxib was confirmed. The purity was determined to be >99% by high-performance liquid chromatography. Antibody for COX-2 was purchased from Cayman Chemical (Ann Arbor, MI). Antibody against CTR1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other primary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Control siRNA, COX-2 siRNA, and HiPerFect Transfection Reagent were from QIAGEN GmbH (Hilden, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**Cell Culture and Viability Assay.** Human esophageal squamous cell carcinoma cell line HKESC-1 was kindly provided by Prof. G. Srivastava (Department of Pathology, The University of Hong Kong, Hong Kong, China). HKESC-1 was established from moderately differentiated human esophageal squamous cell carcinoma (Hu et al., 2000). The cisplatin-resistant subline, designated HKESC-1/cis, was originated by growing parental HKESC-1 cells with gradually increasing doses of cisplatin (from 0.1 to 10  $\mu$ M). HKESC-1 and HKESC-1/cis cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as described previously (Yu et al., 2009).

**Cell Death Detection ELISA Assay for Apoptosis.** Apoptosis was measured using the Cell Death Detection ELISA plus system (Roche Diagnostics Corp., Indianapolis, IN), a spectrophotometric enzyme immunoassay for the in vitro determination of cytoplasmic histone-associated DNA-fragments of mono- and oligonucleosomes after induced cell death.

**Western Blotting.** Cells were harvested in radioimmunoprecipitation buffer containing protease and phosphatase inhibitors as described previously (Yu et al., 2009). Equal amounts of protein (50  $\mu$ g/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) and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

**RNA Interference.** The expression of COX-2 was lowered using predesigned target-specific siRNA oligonucleotides, a method employed in our previous study (Yu et al., 2009). COX-2 siRNA was transfected into cells using HiPerFect Transfection Reagent according to the manufacturer's instructions.

**PGE<sub>2</sub> Assay.** The measurement of PGE<sub>2</sub> in the cell culture medium was carried out by using the Correlate-EIA Prostaglandin E<sub>2</sub> Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions.

**Measurement of Cisplatin Accumulation into Whole Cells and DNA.** Cisplatin accumulation was determined as described previously with some modification (Holzer et al., 2004b; Samimi et al., 2004). For whole-cell platinum accumulation studies, the cells

were incubated in fresh medium containing 30  $\mu\text{M}$  cisplatin alone or in combination with tested COX inhibitors for indicated time points (see Fig. 3). In the case of the time 0 samples, the drug-containing medium was aspirated within 15 s of the start of drug exposure (Blair et al., 2009). The cells were then washed three times with ice-cold PBS and lysed directly by addition of 215  $\mu\text{l}$  of 70% nitric acid into each well. The cells were then collected and dissolved at 65°C for 2 h, after which the samples were diluted with water to a final concentration of 5% acid. For DNA platinum accumulation studies, the cells were incubated in fresh medium containing 30  $\mu\text{M}$  cisplatin for 2 h. The cells were then washed three times with ice-cold PBS before isolation of genomic DNA using a Wizard Genomic DNA Purification kit (Promega, Madison, WI) as recommended by the manufacturer. Harvested DNA was originally resuspended in 70  $\mu\text{l}$  of water for quantification. After quantification, 215  $\mu\text{l}$  of 70% nitric acid was added to the DNA, and the samples were dissolved at 65°C for 2 h. After that, the samples were diluted with water to a final concentration of 5% acid. Platinum content was determined using a Thermo X Series II inductively coupled plasma mass spectroscopy (ICP-MS) and normalized to protein levels or DNA amounts. Lysates from a set of identical cultures were used to measure protein concentrations. Indium was added to each sample at 1 ppb as a control for flow variation.

**Measurement of Cisplatin Export.** Cells were incubated in fresh medium containing 30  $\mu\text{M}$  cisplatin for 2 h and immediately rinsed with ice-cold PBS, after which the cisplatin-containing medium was replaced with fresh medium or medium containing tested COX inhibitors. After 2-h incubation, whole-cell cisplatin accumulation was determined as described under *Measurement of Cisplatin Accumulation into Whole Cells and DNA*.

**Nude Mice Xenograft Model.** HKESC-1 cells were trypsinized and collected by centrifugation. Cell viability was confirmed to be above 95% based on trypan blue staining. The cells ( $2 \times 10^6$ ) were suspended in 0.2 ml of PBS and injected subcutaneously into the right flank or dorsal region of 4- to 6-week-old female BALB/c nu/nu mice (The Animal Center, Sun Yat-Sen University, Guangzhou, China). After inoculation, the mice were maintained under sterile conditions, and the size of tumor formed was measured using calipers every 2 days. Tumor volume was calculated by the following formula: volume = (length/2)  $\times$  (width<sup>2</sup>). On day 10 after inoculation, all mice produced a palpable tumor. The mice were then divided randomly into four groups of eight mice each: 1) control group in which vehicle alone was received; 2) cisplatin-treated group in which 2 mg/kg cisplatin (diluted with physiological saline) was administered intraperitoneally once a week for 4 weeks; 3) celecoxib-treated group in which 100 mg/kg celecoxib (suspended in PBS) was administered by lavage daily for 4 weeks; and 4) cisplatin plus celecoxib-treated group in which 2 mg/kg i.p. cisplatin was administered once a week for 4 weeks, and 100 mg/kg celecoxib was administered by lavage daily for 4 weeks. Body weight was monitored throughout the experiments. At the end of the experiment, the mice were sacrificed and tumors were excised for further assays. Animal care and experiments were conducted in accordance with the Animal Research Committee Guidelines of Nanfang Hospital, Southern Medical University.

**TUNEL Assay for Apoptosis.** Frozen tumor tissues embedded in optimal cutting temperature compound (Tissue-Tek O.C.T. Compound; Sakura Finetech USA, Torrance, CA) were sectioned at 10  $\mu\text{M}$  thickness. To identify apoptosis in tumor sections, the TUNEL method was applied, using the In Situ Cell Death Detection kit (Roche Diagnostics Corp.) according to manufacturer's instructions. Apoptotic cells were visualized under an Olympus fluorescence microscope equipped with a computer-controlled digital camera. TUNEL-positive (apoptotic) and 4,6-diamidino-2-phenylindole-stained (total) nuclei were counted using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD).

**Statistical Analysis.** Results were expressed as mean  $\pm$  S.E.M. Statistical analysis was done by using Prism statistical software

(GraphPad Software, San Diego, CA). Student's two-tailed *t* test was used to compare data between two groups. Both one-way ANOVA and subsequently Tukey's *t* test were used to compare data between three or more groups. *P* values less than 0.05 were considered statistically significant.

## Results

**Celecoxib and SC-236 Antagonized Cisplatin-Induced Cytotoxicity in Esophageal Squamous Cell Carcinoma Cell Lines.** To study whether COX inhibitors could influence the cytotoxic action of cisplatin, HKESC-1 and its cisplatin-resistant subline HKESC-1/cis were treated with cisplatin (0–160  $\mu\text{M}$ ) in the absence or presence of any one of the following COX inhibitors: celecoxib, SC-236, nimesulide, or indomethacin. All the COX inhibitors were used at a concentration of 20  $\mu\text{M}$ . Celecoxib, SC-236, and nimesulide are COX-2-selective inhibitors, whereas indomethacin is a nonselective NSAID. As shown in Table 1, HKESC-1/cis was 2.9 times more resistant to cisplatin-induced cytotoxicity than the parental cell line HKESC-1. Cotreatment of HKESC-1 or HKESC-1/cis cells with celecoxib or SC-236, neither of which alone had any effect on cell viability, increased the IC<sub>50</sub> value of cisplatin. In contrast, the other tested COX-2-selective inhibitor nimesulide and the NSAID indomethacin exerted no effect on the cytotoxic action of cisplatin in either cell line.

To determine the minimal effective concentrations of celecoxib and SC-236, HKESC-1 cells were incubated with various concentrations of celecoxib (0.31, 1.25, 5, and 20  $\mu\text{M}$ ) or SC-236 (0.31, 1.25, 5, and 20  $\mu\text{M}$ ) in combination with 5  $\mu\text{M}$  cisplatin, which was the approximate IC<sub>50</sub> for HKESC-1 cells. Both celecoxib and SC-236 significantly antagonized the cytotoxic action of cisplatin at a concentration as low as 5  $\mu\text{M}$ . Likewise, under the same concentration, these two COX-2-selective inhibitors significantly antagonized HKESC-1/cis cells to the cytotoxicity of cisplatin (15  $\mu\text{M}$ , around the IC<sub>50</sub> for HKESC-1/cis cells) (data not shown).

**Antagonizing Effect of Celecoxib or SC-236 on Cisplatin-Induced Cytotoxicity Was Not Associated with COX Inhibitory Activity.** Only celecoxib and SC-236, among the four tested COX inhibitors, showed antagonizing effects on cisplatin-induced cytotoxicity. Therefore, we investigated whether this result could be attributed to their different COX inhibitory activity. COX inhibitory activity was determined by measuring the level of PGE<sub>2</sub>,

TABLE 1

Effects of COX-2 inhibitors on cisplatin-induced cytotoxicity in HKESC-1 and its cisplatin-resistant subline HKESC-1/cis

HKESC-1 cells or HKESC-1/cis cells were treated for 48 h with cisplatin (0–160  $\mu\text{M}$ ) alone or in combination with celecoxib (20  $\mu\text{M}$ ), SC-236 (20  $\mu\text{M}$ ), nimesulide (20  $\mu\text{M}$ ), or indomethacin (20  $\mu\text{M}$ ). Cell viability was then determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. IC<sub>50</sub> values were calculated with Prism software. Data are presented as mean  $\pm$  S.E.M.

Drugs	IC <sub>50</sub>	
	HKESC-1	HKESC-1/cis
	$\mu\text{M}$	
Cisplatin	4.5 $\pm$ 0.2	12.9 $\pm$ 0.4
+ Celecoxib	15.2 $\pm$ 0.2	32.1 $\pm$ 1.2
+ SC-236	10.8 $\pm$ 1.0	31.4 $\pm$ 1.7
+ Nimesulide	5.2 $\pm$ 0.5	15.5 $\pm$ 0.9
+ Indomethacin	4.3 $\pm$ 0.5	15.8 $\pm$ 1.9



the COX end product, released from HKESC-1 cells in response to treatment with COX inhibitors. As shown in Fig. 1A, nimesulide and indomethacin suppressed PGE<sub>2</sub> release to a degree similar to that of celecoxib or SC-236. However, unlike celecoxib or SC-236, these two COX inhibitors exerted no effect on the cytotoxic action of cisplatin in cancer cells. When celecoxib was tested at four concentrations (0.31, 1.25, 5, and 20  $\mu$ M), a significant decrease in PGE<sub>2</sub> release was observed, but to an equal degree, indicating that there was no difference among the concentrations (Fig. 1B). It should be noted, however, that celecoxib antagonized the cytotoxicity of cisplatin at concentrations of 5 and 20  $\mu$ M but not at 0.31 and 1.25  $\mu$ M. Similar results were observed in cells treated with SC-236 (Fig. 1C). These findings suggest that the antagonizing effect of celecoxib and SC-236 on the cytotoxicity of cisplatin did not result from their COX inhibitory activity.

**Down-Regulation of COX-2 Expression Could Not Antagonize Cisplatin-Induced Cytotoxicity in HKESC-1 Cells.** Because the three tested COX-2-selective inhibitors showed inconsistent effect on cisplatin-induced cytotoxicity, the direct role of COX-2 in this action was further investigated by RNA interference experiments. The efficacy of COX-2 depletion by specific siRNA was verified by Western blot analysis (Fig. 2A). In relation to the role of COX-2 in the cytotoxic action of cisplatin, Fig. 2B showed that down-regulation of COX-2 could not antagonize cisplatin-induced cytotoxicity, indicating that inhibition of COX-2 expression in cancer cells is not required for the antagonizing effect of celecoxib or SC-236.

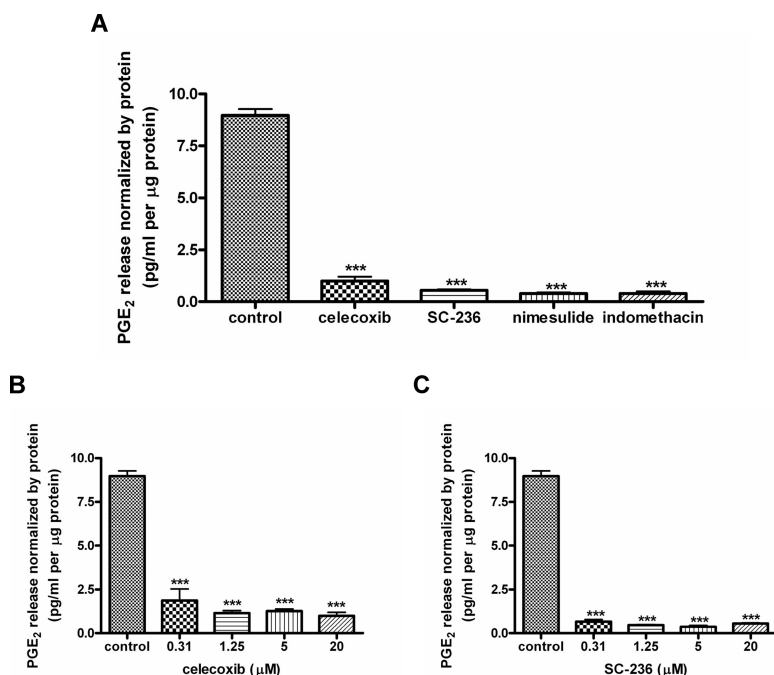
**Celecoxib Antagonized Cisplatin-Induced Apoptosis in Both HKESC-1 and HKESC-1/cis Cells.** Cisplatin is primarily considered a DNA-damaging antitumor drug. Its cytotoxic mode of action is thought to be mediated by its interaction with DNA to form platinum-DNA adducts, which culminate in the activation of apoptosis (Kelland, 2007). Therefore, reduction of cisplatin-induced apoptosis may contribute to the antagonizing effect of celecoxib on the cytotoxic action of cisplatin. To verify this hypothesis, a classic hallmark of apopto-

sis, the cleavage of poly(ADP-ribose) polymerase (PARP), was therefore examined (Fig. 3A). Celecoxib alone showed no effect on the cleavage of PARP, but it obviously attenuated cisplatin-induced cleavage of PARP in both HKESC-1 and HKESC-1/cis cells.

To better quantitatively determine the antagonizing effect of celecoxib on cisplatin-induced apoptosis, a Cell Death Detection ELISA<sup>PLUS</sup> kit from Roche was used to detect mono- and oligonucleosomes that are released into the cytoplasm in cells undergoing apoptosis. As shown in Fig. 3B, celecoxib at a concentration as low as 5  $\mu$ M significantly antagonized cisplatin-induced apoptosis in both HKESC-1 cells and HKESC-1/cis cells, which agreed with the antagonizing effect of celecoxib on cisplatin-induced cytotoxicity.

**Celecoxib and SC-236 Reduced Whole-Cell Cisplatin Accumulation.** It has been consistently demonstrated that accumulation of cisplatin is a determinant of cellular sensitivity (Hall et al., 2008). To determine whether the antagonizing effect of celecoxib and SC-236 on cisplatin-induced cytotoxicity was linked to changes in cisplatin accumulation, total whole-cell platinum accumulation was measured after a 2-h exposure to 30  $\mu$ M cisplatin in the absence or presence of COX inhibitors in HKESC-1 cells by ICP-MS. Figure 4A showed that celecoxib and SC-236 significantly reduced the whole-cell accumulation of cisplatin, whereas nimesulide and indomethacin exerted no effect. The accumulation curve further indicated that celecoxib decreased intracellular cisplatin content within 10 h of treatment (Fig. 4B).

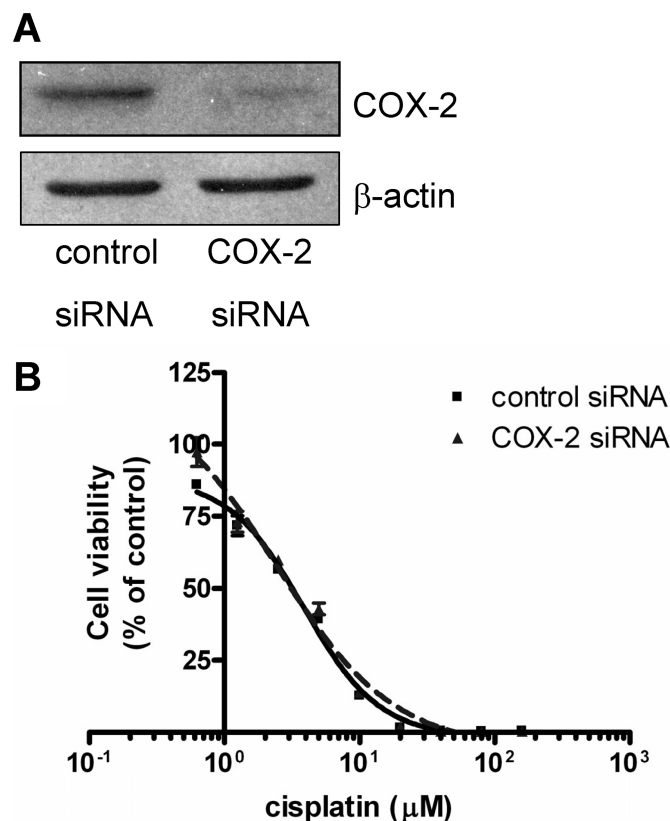
To further determine the minimal effective concentrations of celecoxib and SC-236 on the whole-cell accumulation of cisplatin, HKESC-1 cells were incubated with various concentrations of celecoxib or SC-236 in combination with 30  $\mu$ M cisplatin for 2 h. At a concentration as low as 5  $\mu$ M, both celecoxib and SC-236 significantly reduced whole-cell cisplatin accumulation (Fig. 4, C and D). The fact that the reduction of whole-cell cisplatin accumulation induced by celecoxib or SC-236 closely paralleled their antagonizing effects on cisplatin-induced cytotoxicity indi-



**Fig. 1.** Effects of COX inhibitors on PGE<sub>2</sub> release from HKESC-1 cells. Cells were treated with indicated COX inhibitors (20  $\mu$ M celecoxib, 20  $\mu$ M SC-236, 20  $\mu$ M nimesulide, or 20  $\mu$ M indomethacin) (A), various concentrations of celecoxib (B), or SC-236 (C) for 48 h. Supernatants were then collected for PGE<sub>2</sub> measurement. The PGE<sub>2</sub> level was expressed as picograms per milliliter per microgram of protein. Data are represented as the mean  $\pm$  S.E.M. ( $n = 3$ ) of a representative experiment performed in triplicate. \*\*\*,  $p < 0.001$  compared with control group.

cates that celecoxib and SC-236 antagonized the cytotoxic action of cisplatin by reducing whole-cell cisplatin accumulation. Moreover, it is likely that celecoxib and SC-236 reduce intracellular cisplatin accumulation in a COX-2-independent manner, for nimesulide and indomethacin exerted no effect on intracellular drug accumulation.

**Celecoxib and SC-236 Reduced DNA Adduct Formation.** Cisplatin that enters the cell works by binding to DNA and forming DNA adducts that lead primarily to cellular apoptosis (Kelland, 2007). Therefore, the cytotoxicity of cisplatin is proportional to the amount of drug entering the cell that actually forms adducts in DNA. To determine whether the reduced intracellular accumulation of cisplatin led to less drug reaching the nucleus, which is the critical target mediating cytotoxicity, the extent of DNA adduct formation was measured. As shown in Fig. 4E, celecoxib or SC-236 significantly reduced the formation of DNA adduct, whereas nimesulide and indomethacin exerted no effect. The attenuating effects of celecoxib or SC-236 on intracellular cisplatin accumulation and cisplatin-DNA adduct formation were comparable, suggesting that the cisplatin entering the cell represents a pool of drug available for trafficking to the nucleus and forming DNA adducts.

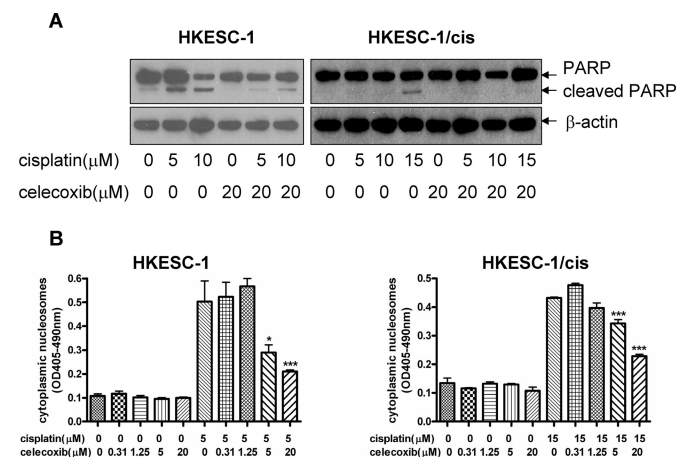


**Fig. 2.** Effects of siRNA-mediated knockdown of COX-2 on cisplatin-induced cytotoxicity in HKESC-1 cells. **A**, the efficacy of COX-2 depletion by COX-2 siRNA was verified by Western blot analysis. Nontargeting siRNA was used as control siRNA, which has no homology to any known mammalian genes.  $\beta$ -Actin was used to evaluate protein loading. These results are representative of three independent experiments. **B**, after transfection with the control siRNA or COX-2 siRNA, cells were treated with cisplatin at indicated concentrations for 48 h before 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Data are represented as the mean  $\pm$  S.E.M. ( $n = 3$ ) of a representative experiment performed in triplicate.

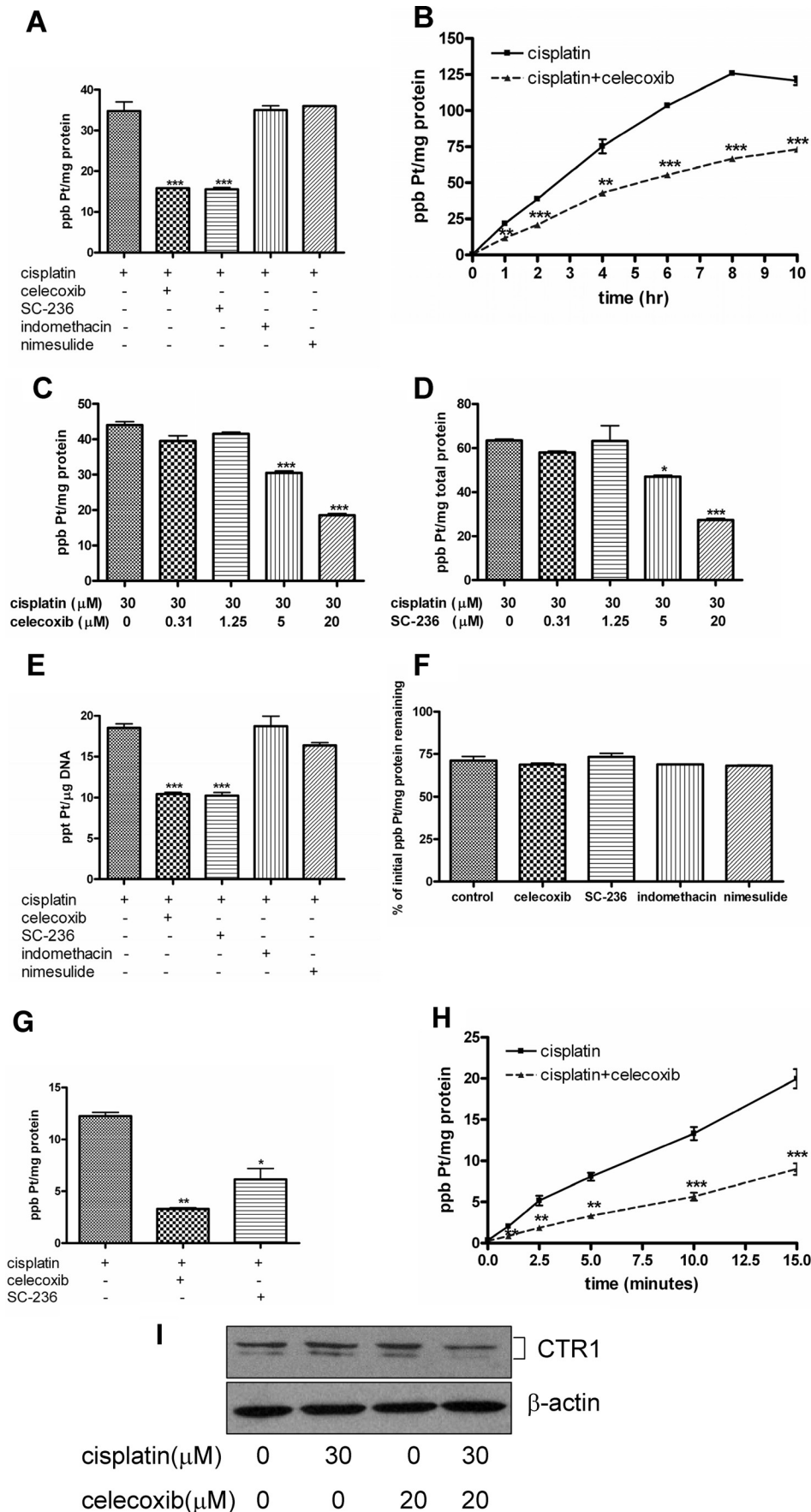
**Celecoxib Reduced Intracellular Cisplatin Accumulation by Decreasing Drug Influx.** The effect of celecoxib on the intracellular accumulation of cisplatin in cells exposed to drugs for 2 h did not provide information concerning whether the reduced intracellular cisplatin was caused by decreased drug influx, enhanced efflux, or a combination of both. Therefore, cisplatin export was determined. Figure 4F shows the percentage of cisplatin remaining in cells. After withdrawal of cisplatin for 2 h,  $71.2 \pm 2.3\%$  of previously accumulated cisplatin was left in cells. None of the tested COX inhibitors changed intracellular cisplatin retention compared with control, indicating that the tested COX inhibitors did not change the export of cisplatin. Therefore, the attenuating effect of celecoxib or SC-236 on intracellular cisplatin accumulation is not related to their effects on cisplatin export.

To determine whether reduced drug influx contributed to the lowered cisplatin accumulation, short-term cisplatin accumulation experiments were conducted. In general, when a change in drug accumulation is observed on the initial phase (5 min), this can be interpreted as indicating an effect on the influx step (Blair et al., 2009; Larson et al., 2009). As shown in Fig. 4G, cells treated with cisplatin in combination with celecoxib or SC-236 for 5 min contained much less cisplatin than those treated with cisplatin alone. The early accumulation kinetics of cisplatin with and without celecoxib was further determined by measurement of intracellular cisplatin accumulation from 0 to 15 min. As shown in Fig. 4H, celecoxib obviously reduced cisplatin accumulation within 15 min of treatment. These results suggest that celecoxib reduces intracellular cisplatin accumulation by decreasing cisplatin influx.

**The Combination of Celecoxib with Cisplatin Reduced the Protein Expression of CTR1.** Cisplatin cannot readily diffuse across the cellular membrane because of its



**Fig. 3.** Celecoxib antagonized cisplatin-induced apoptosis in both HKESC-1 and HKESC-1/cis cells. **A**, after treatment of HKESC-1 cells or HKESC-1/cis cells with various concentrations of cisplatin in the absence or presence of celecoxib (20  $\mu$ M) for 48 h, the effect of celecoxib on cisplatin-induced PARP cleavage was examined by Western blot.  $\beta$ -Actin was used to evaluate protein loading. These results are representative of three independent experiments. **B**, HKESC-1 cells or HKESC-1/cis cells were treated with or without cisplatin in the presence or absence of various concentrations of celecoxib as indicated for 48 h, and the cytosolic content of mono- and oligonucleosomes was determined by ELISA. Data are represented as the mean  $\pm$  S.E.M. ( $n = 3$ ) of a representative experiment performed in triplicate. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  compared with cisplatin-treated group.



**Fig. 4.** Effects of COX inhibitors on cisplatin accumulation in cells and in DNA. A, HKESC-1 cells were treated with 30  $\mu$ M cisplatin in the absence or presence of COX inhibitors for 2 h. All the COX inhibitors were used at a concentration of 20  $\mu$ M. The intracellular cisplatin accumulation was then measured by ICP-MS. B, HKESC-1 cells were treated with 30  $\mu$ M cisplatin in the absence or presence of 20  $\mu$ M celecoxib. The intracellular cisplatin accumulation was measured by ICP-MS after 0, 1, 2, 4, 6, 8, and 10 h of drug exposure. C and D, HKESC-1 cells were treated with cisplatin (30  $\mu$ M) alone or in combination with various concentrations of celecoxib or SC-236 for 2 h. The intracellular cisplatin accumulation was then measured by ICP-MS. E, HKESC-1 cells were treated with 30  $\mu$ M cisplatin in the absence or presence of COX inhibitors (celecoxib, SC-236, nimesulide, or indomethacin) for 2 h. All the COX inhibitors were used at a concentration of 20  $\mu$ M. The cisplatin accumulation in DNA was then measured by ICP-MS. F, HKESC-1 cells were treated with cisplatin (30  $\mu$ M) for 2 h followed by 2-h incubation in normal growth medium or medium containing COX inhibitors (celecoxib, SC-236, nimesulide, or indomethacin). All the COX inhibitors were used at a concentration of 20  $\mu$ M. The intracellular cisplatin retention was then determined by ICP-MS. G, HKESC-1 cells were treated with 30  $\mu$ M cisplatin alone or in combination with 20  $\mu$ M celecoxib or 20  $\mu$ M SC-236 for 5 min, then the intracellular cisplatin accumulation was measured by ICP-MS. H, HKESC-1 cells were treated with 30  $\mu$ M cisplatin in the absence or presence of 20  $\mu$ M celecoxib. The intracellular cisplatin accumulation was measured by ICP-MS after 0, 1, 2.5, 5, 10, and 15 min of drug exposure. Data are represented as the mean  $\pm$  S.E.M. ( $n = 3$ ) of a representative experiment performed in triplicate. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$  compared with cisplatin-treated group. I, Cells were incubated for 5 min with 30  $\mu$ M cisplatin, 20  $\mu$ M celecoxib, and their combination. Cells were then collected for the determination of CTR1 expression by Western blot analysis.  $\beta$ -Actin was used to evaluate protein loading. These results are representative of three independent experiments.



high polarity (Hall et al., 2008; Howell et al., 2010). The major copper influx transporter, copper transporter 1 (CTR1), has been shown to mediate the cisplatin influx in mammalian cells (Holzer et al., 2004a; Holzer and Howell, 2006; Howell et al., 2010). Therefore, we determined the protein expression of CTR1 in HKESC-1 cells in response to the exposure of cisplatin, celecoxib, and their combination. As shown in Fig. 4I, the combination of cisplatin and celecoxib reduced the protein expression of CTR1, suggesting that the lowered CTR1 may lead to the reduced cisplatin influx.

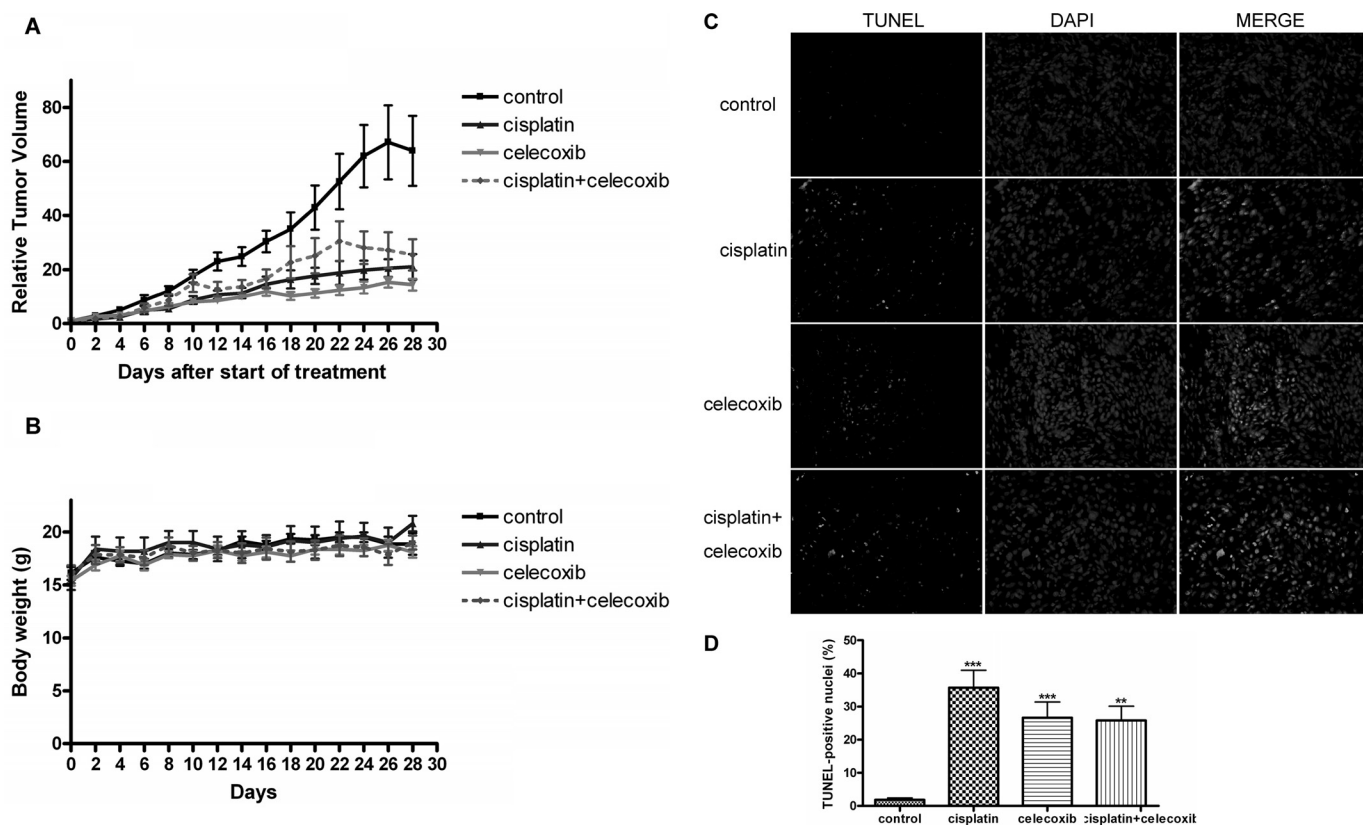
**The Combination of Celecoxib with Cisplatin Failed to Enhance the Growth Inhibition Activity of Celecoxib or Cisplatin Alone In Vivo.** The antitumor activity of cisplatin, celecoxib, and their combination in vivo was evaluated using a 28-day esophageal squamous cell carcinoma xenograft model in nude mice. In this study, the growth rate of the tumor was significantly retarded by the treatment of cisplatin ( $p < 0.01$ ) or celecoxib ( $p < 0.001$ ) alone compared with control group (Fig. 5A). Although the combination of cisplatin and celecoxib also demonstrated a statistically significant reduction of tumor growth rate compared with control group ( $p < 0.05$ ), the combination of these two drugs did not elicit greater antitumor effect compared with either cisplatin ( $p > 0.05$ ) or celecoxib alone ( $p > 0.05$ ). Moreover, the combination treatment tended to attenuate the antitumor

effect of cisplatin or celecoxib monotherapy. No obvious systemic toxicity was observed during the entire period of drug treatment based on the body weight data (Fig. 5B).

**The Combination of Celecoxib with Cisplatin Did Not Enhance Celecoxib or Cisplatin-Induced Apoptosis In Vivo.** In general, antitumor drugs inhibit tumor growth mainly through induction of apoptosis on tumor cells. Therefore, we investigated the effects of the combined celecoxib and cisplatin treatment or either agent alone on apoptosis induction in the tumor sections. TUNEL assays were used to evaluate cellular apoptosis. Figure 5C showed representative images from each group, and Fig. 5D showed the average percentage of TUNEL-positive cells in each group. Quantification of apoptotic cells within the tumor sections revealed that the combination of celecoxib and cisplatin or either agent alone significantly increased apoptosis in the tumors in comparison with control group. However, the combined treatment did not enhance celecoxib or cisplatin-induced apoptosis.

## Discussion

Some correlations have been reported between reduced cisplatin-based therapy efficacy and high COX-2 expression in patients with esophageal cancer (Takatori et al., 2005; Xi



**Fig. 5.** Effects of cisplatin, celecoxib, and their combination on the growth of esophageal squamous cell carcinoma xenografts in nude mice. A, HKESC-1 cells ( $2 \times 10^6$ ) in 0.2 ml of PBS were injected subcutaneously into the right flank or dorsal region of 4- to 6-week-old female BALB/c nu/nu mice. After tumors reached a palpable size, the mice were divided randomly into four groups of eight mice each and treated with cisplatin, celecoxib, and their combination for 28 days. The starting day of drug treatment was defined as day 0. B, body weight changes in mice during the period of treatment. Treatment with cisplatin, celecoxib, and their combination did not significantly affect body weight compared with control group. Data are presented as mean  $\pm$  S.E.M. C, representative fluorescence images from each group. D, the percentage of TUNEL-positive cells was assessed on sections from xenografts from each group. Percentage of apoptotic cells was calculated by dividing the number of apoptotic cells with the total number of cells counted from 10 random microscopic fields per sample at the  $400\times$  magnification. Data are shown for four animals/group and presented as mean  $\pm$  S.E.M. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with control group.

et al., 2005). However, the therapeutic benefit of including NSAIDs or COX-2-selective inhibitors in cisplatin-based treatment regimens is poorly defined (Tew et al., 2005; Dawson et al., 2007). Moreover, the effects of NSAIDs or COX-2-selective inhibitors on cisplatin-induced cytotoxicity in human esophageal squamous cell carcinoma cells have not been reported. Therefore, we investigated the role of NSAIDs and COX-2-selective inhibitors in the cytotoxic action of cisplatin. Our results show that the COX-2-selective inhibitors celecoxib and SC-236 antagonize the cytotoxicity of cisplatin in human esophageal squamous cell carcinoma cells and their cisplatin-resistant counterparts. Coadministration of celecoxib and cisplatin did not enhance the antitumor activity of the drugs given alone in an *in vivo* esophageal squamous cell carcinoma xenograft model in mice. Instead there was a trend that such combination of drugs had a lesser antitumor activity than the respective drugs administered alone.

Although celecoxib and SC-236 are COX-2-selective inhibitors, we present evidence that their antagonizing effect on the cytotoxicity of cisplatin is COX-2-independent. First, the nonselective NSAID indomethacin and the other tested COX-2-selective inhibitor nimesulide showed no influence on cytotoxic action of cisplatin, although all of the tested COX inhibitors suppressed PGE<sub>2</sub> production to an equal degree. In addition to the pharmacological approach, siRNA-mediated knockdown of COX-2 also showed no effect on the cytotoxic action of cisplatin, which provides the direct evidence for a COX-2-independent mechanism. Finally, chemical structures of the tested compounds also offer indirect evidence supporting our conclusion. In this respect, SC-236 is a structural analog of celecoxib. The only difference between these two compounds is that the 4-chloro substituent on SC-236 is replaced by a methyl group in celecoxib. In contrast, the structure of nimesulide or indomethacin is very different from that of celecoxib. Therefore, it seems that the antagonizing effect of celecoxib and SC-236 results from their special chemical structures rather than their COX-2 inhibitory activity. Consistent with our findings, it was also demonstrated that celecoxib antagonized cisplatin-induced cytotoxicity in a COX-2-independent manner in a panel of human ovarian cancer cell lines in which COX-2 expression was undetectable (Bijman et al., 2008).

Multiple lines of evidence indicate that the cytotoxicity of cisplatin is directly related to how much drug enters the cell, and cisplatin-resistant cells uniformly tend to contain less cisplatin than their wild-type counterparts (Hall et al., 2008). In agreement with these findings, our results show that the cisplatin-resistant subline HKESC-1/cis exhibited reduced drug accumulation compared with its parent cell line HKESC-1 (data not shown). In light of the correlation between intracellular accumulation of cisplatin and its cytotoxicity, we then asked whether decreased accumulation of cisplatin might account for the antagonizing effects of celecoxib or SC-236 on the cytotoxic action of cisplatin. Analysis of whole-cell cisplatin accumulation demonstrated that HKESC-1 cells, which were cotreated with cisplatin and celecoxib or SC236, contained less cisplatin than those treated with cisplatin alone. Moreover, the minimal effective concentrations of celecoxib or SC-236 required to reduce intracellular cisplatin accumulation are consistent with those required to inhibit cisplatin-induced cytotoxicity and apoptosis. Because DNA damage is

thought to be the primary mechanism by which cisplatin exerts its cytotoxicity, the extent of cisplatin-DNA adduct formation was further determined in our study. There was less cisplatin-adduct formation in cisplatin-resistant cell line HKESC-1/cis than its wild-type counterpart HKESC-1 (data not shown). More noteworthy are our results showing that celecoxib or SC-236 also decreased the extent of DNA adduct formation. This decrease is similar to the magnitude of the change in intracellular cisplatin accumulation. In contrast, indomethacin and nimesulide, unlike celecoxib and SC-236, exerted no influence on either intracellular cisplatin accumulation or DNA adduct formation, suggesting that reduced cellular accumulation of cisplatin does not result from the COX-2 inhibitory activity of celecoxib or SC-236.

In theory, decreased cellular accumulation of cisplatin could be caused by either reduced influx, enhanced efflux, or a combination of the two. Celecoxib or SC-236 did not change the intracellular retention of cisplatin after withdrawal of cisplatin, indicating that decreased accumulation does not result from enhanced drug efflux. In contrast, a reduction in accumulation was observed on the initial phase (5 min), which was interpreted as indicating an effect on influx (Blair et al., 2009; Larson et al., 2009). These findings suggest that celecoxib and SC-236 reduce intracellular cisplatin accumulation by decreased drug influx rather than enhanced efflux. CTR1 is a major determinant of initial influx of cisplatin, as loss of CTR1 reduced influx measured over the first 5 min of drug exposure by 81% (Larson et al., 2009). Although cisplatin-induced rapid down-regulation of CTR1 has been documented in some cell types (Holzer et al., 2004a; Holzer and Howell, 2006), our studies show that CTR1 protein level does not change in response to a 5-min exposure to cisplatin. However, short-term exposure to cisplatin in the presence of celecoxib decreased CTR1 protein expression, suggesting that the reduction of protein level of CTR1 may mediate, at least in part, the reduced influx of cisplatin induced by the combination treatment. Because experiments (<sup>1</sup>H NMR) have excluded a direct interaction between celecoxib and cisplatin (Bijman et al., 2008), it is unlikely that the interaction between cisplatin and celecoxib contributes to decreased CTR1 expression. The mechanistic study indicates that cisplatin-induced reduction of CTR1 involves internalization from the plasma membrane by macropinocytosis followed by proteasomal degradation (Holzer and Howell, 2006). Whether the combination treatment decreased CTR1 expression through accelerating the rate of cisplatin-induced CTR1 degradation or other mechanisms needs further exploration.

In addition to our *in vitro* study investigating the action of celecoxib on the cytotoxicity of cisplatin, we also determined the outcome of combined treatments of cisplatin with celecoxib *in vivo*. The growth rate of tumor was significantly retarded by the treatment of cisplatin or celecoxib alone. Unexpectedly, the outcome of combined treatment did not show additive effects between cisplatin and celecoxib. In addition, there was no additive effect between these two drugs on apoptosis induction. The compromised cisplatin efficacy by coadministration of celecoxib may be responsible, at least in part, for this result, if we take into account the drug concentration. The plasma concentration in nude mice was 3 to 7  $\mu$ M at 1 h after injection of 2 mg/kg cisplatin (Johnsson et al., 1995). This concentration is comparable with that used in our *in vitro* study. With respect to the dose of celecoxib, it



has been reported that the plasma concentration of celecoxib in nude mice was  $3.8 \pm 0.7 \mu\text{M}$  between 2 and 4 h after administration of celecoxib (Grösch et al., 2001). It is noteworthy that the administration of celecoxib to mice in the above study (100 mg/kg per day for 3 weeks) is almost the same as the protocol used in our study (100 mg/kg per day for 4 weeks). With respect to our in vitro data, the minimal effective concentration of celecoxib to antagonize cytotoxicity of cisplatin and to reduce intracellular cisplatin accumulation is  $5 \mu\text{M}$ , which is very close to the reported plasma concentration. Therefore, celecoxib may compromise anti-tumor activity of cisplatin by decreasing intracellular cisplatin accumulation when these two drugs are administered simultaneously.

In conclusion, our studies show, for the first time, that celecoxib antagonizes the cytotoxicity and proapoptotic activity of cisplatin by decreasing intracellular cisplatin accumulation and the extent of cisplatin-DNA adduct formation in human esophageal squamous cell carcinoma cells. The antagonizing effects of celecoxib are independent of COX-2 inhibition but associated with the reduced cisplatin influx that is accompanied by the reduction of CTR1 protein level. The combined treatment of celecoxib with cisplatin also shows no beneficial effect compared with celecoxib or cisplatin alone in vivo. It is even more noteworthy that the minimal effective concentration of celecoxib ( $5 \mu\text{M}$ ) to antagonize cisplatin cytotoxicity is in the concentration range found in human plasma after administration of celecoxib ( $3\text{--}5 \mu\text{M}$ ) (Niederberger et al., 2001). Based on these findings, current clinical trials with celecoxib in combination with cisplatin should be approached with caution.

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## Authorship Contributions

*Participated in research design:* Yu, Jiang, Wu, Cho, Liu.

*Conducted experiments:* Yu, Chen, Li.

*Contributed new reagents or analytic tools:* Wen, Fu, Guo.

*Performed data analysis:* Yu.

*Wrote or contributed to the writing of the manuscript:* Yu, Jiang, Wu, Cho, Liu.

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