

# Celecoxib exerts antitumor effects in canine mammary tumor cells via COX-2-independent mechanisms

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**Abstract.** Celecoxib plays antitumor roles via multiple mechanisms in a variety of human cancers. The aim of this study was to clarify the mechanism of action of celecoxib in canine mammary tumors. We examined the antitumor effects of celecoxib in AZACB canine mammary tumor cells expressing low levels of cyclooxygenase-2 (COX-2) to minimize the effect of COX-2 on its activity. Our data revealed that celecoxib inhibited cell proliferation mainly via COX-2-independent mechanisms. Specifically, celecoxib decreased the proportion of cells in S phase and increased G2/M arrest, which was associated with increased expression of the cyclin-dependent kinase inhibitors (CDKIs) p21 and p27. In addition, treatment with celecoxib downregulated COX-2 expression, and induced apoptosis via both the intrinsic and extrinsic pathways. These findings suggest that celecoxib might be a useful agent for the treatment of canine mammary tumors, regardless of COX-2 expression. In the future, it might be possible to use a combination of celecoxib and other antitumor agents to treat canine mammary tumors.

## Introduction

Arachidonic acids are converted into prostaglandin (PG) H<sub>2</sub>, which is the precursor of eicosanoids, including PGs, prostacyclin (PGI<sub>2</sub>), and thromboxanes (TXs), in a reaction that is catalyzed by cyclooxygenases (COXs). There are three COX isoenzymes: COX-1, -2, and -3. COX-1 is constitutively expressed in most tissues, and plays an important role in protecting the gastric mucosa, regulating platelet aggregation, and maintaining renal blood flow (1). COX-3

was initially identified as an alternatively spliced variant of COX-1 in dogs (2). Although COX-3 is a potential target of the antipyretic and analgesic effects of acetaminophen, its detailed function remains unclear (2). In contrast, it is known that cyclooxygenase-2 (COX-2) is induced by various stimuli such as pro-inflammatory cytokines during inflammation or the initiation and progression of cancer. In cancer, it is suspected that COX-2 plays an important role in angiogenesis, invasion, apoptosis resistance, immune evasion, and drug resistance (3-9). Therefore, it might play a role in the antitumor effects of non-steroidal anti-inflammatory drugs (NSAIDs). Selective COX-2 inhibitors have been developed to reduce gastrointestinal dysfunction, and for chemotherapy or chemoprevention in various human cancers. The antitumor effects of selective COX-2 inhibitors are exerted via diverse means, including COX-2-dependent and -independent mechanisms and the activation of intrinsic and extrinsic apoptotic pathways. However, the detailed mechanism of action of the antitumor effects of selective COX-2 inhibitors in various cancers remains controversial.

Canine mammary tumors are the most common tumors in female dogs without contraception, and approximately half of all cases are malignant. However, it is difficult to diagnose malignant canine mammary tumors histopathologically (10). Accordingly, several studies have explored molecular markers to diagnose or treat malignant canine mammary tumors. Among these, COX-2 has received significant attention as a diagnostic and therapeutic target (11-13). Furthermore, previous reports suggested that canine mammary tumors could be a suitable model for studying human breast cancer (14).

COX-2 overexpression has been reported in human breast cancer (15). Similarly, COX-2 expression was elevated in canine mammary tumors compared with normal mammary tissue (16). In particular, a previous study demonstrated that although no expression was detected in the normal mammary gland, COX-2 was expressed in 56 and 24% of adenocarcinoma and adenoma samples, respectively (16). This report also suggested that COX-2-positive tumor cells might have a higher malignant tendency (16). Furthermore, a previous study reported a correlation between vascular endothelial growth factor (VEGF) and COX-2 levels; the enhanced production of VEGF resulted in increased intra-tumoral microvessel density (17). These findings suggest that COX-2

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might be a potential marker for poor prognosis and a target for chemotherapy in canine mammary tumors. We previously demonstrated the usefulness of selective COX-2 inhibitors as therapeutic agents in canine mammary tumor (CF33) cells, which express high levels of COX-2 (18). However, the detailed mechanism of action of celecoxib in canine mammary tumor is still not completely understood.

Our previous study suggested that selective COX-2 inhibitors exert potential antitumor effects via both COX-2-dependent and -independent mechanisms (18). Accordingly, the aim of this study was to explore the detailed mechanism of action of selective COX-2 inhibitors in canine mammary tumor cells. We analyzed their antitumor effects in AZACB canine mammary tumor cells, which express low levels of COX-2, to minimize the effect of COX-2. Furthermore, we used three different inhibitors (celecoxib, etodolac, and meloxicam) that are highly selective for COX-2; these inhibitors have potential clinical utility as analgesics and anti-inflammatory agents in osteoarthritic dogs (19,20).

## Materials and methods

**Chemicals.** We used meloxicam, etodolac, and celecoxib to assess the antitumor effect of selective COX-2 inhibitors. Celecoxib and etodolac were purchased from Sigma-Aldrich (Tokyo, Japan), and meloxicam was obtained from Wako Pure Chemicals Industries, Ltd., (Osaka, Japan). 2,5-Dimethyl-celecoxib (DMC), a structural analog of celecoxib, was purchased from Sigma-Aldrich. All the drugs were dissolved in 100% DMSO (Wako Pure Chemicals Industries, Ltd.) at different concentrations and stored at -20°C. Control cells were treated with DMSO at a final concentration of 0.1%, whereas parent cells were untreated. The following antibodies were used in the current study: anti-COX-2 (Abcam, Tokyo, Japan), anti- $\beta$ -actin (Sigma-Aldrich), anti-p27 kip1 (BD Transduction Laboratories, Tokyo, Japan), anti-Bax (Millipore, Billerica, MA, USA), and anti-Bid (Abnova, Taipei, Taiwan). All other antibodies were purchased from Cell Signaling Technology, Inc. (Tokyo, Japan). The inhibitors caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) were obtained from R&D Systems (Minneapolis, MN, USA), and were dissolved in 100% DMSO and stored at -20°C.

**Cell lines and culture conditions.** AZACB cells were purchased from Primary Cell Co., Ltd. (Hokkaido, Japan), CF33, and CF41.MG cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, 10 mg/ml streptomycin, and 10,000 U/ml penicillin G at 37°C in a 5% CO<sub>2</sub> incubator. AZACB, CF33, and CF41.MG cells were cultured as described previously (18,21,22).

**Western blotting.** Cells were lysed in radioimmunoprecipitation assay buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% sodium deoxycholate, and various protease inhibitors (1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1  $\mu$ g/ml aprotinin, 1 mM dithiothreitol,

1 mM NaVO<sub>4</sub>, and 0.5 mM phenylmethylsulfonyl fluoride). Whole cell lysates were prepared as described previously (23). The protein concentrations of the cell lysates were then quantified using the Bradford method with a Pierce® BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Total cell lysates (15-25  $\mu$ g) were boiled for 5 min in 2X Laemmli sample loading buffer, and were then separated by SDS-PAGE on 12% (p27, Bax, Bim, and Bid) and 10% (COX-2) gels. The gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Tokyo, Japan). The expression of specific proteins was detected by electrochemiluminescence using WesternBright Quantum or WesternBright Sirius (Advansta, Menlo Park, CA, USA), and observed using ChemiDoc XRS (Bio-Rad Laboratories).

**Cell viability assays.** AZACB and CF33 cells were plated into 96-well plates (BD Falcon; Nippon Becton Dickinson, Tokyo, Japan) at a density of 2.5x10<sup>3</sup> and 1x10<sup>3</sup> cells/well, respectively. After 24 h, the cells were treated with different concentrations of selective COX-2 inhibitors or DMC. Twenty-four hours after treatment, cell number was determined using a WST-8 assay (Cell Counting kit-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a Benchmark Plus microplate reader (Bio-Rad Laboratories). The experiment was performed using five replicates. As a control, it was confirmed that there were no changes in cell viability before drug treatment (day 0).

**Measurements of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).** AZACB cells were plated into 100-mm tissue culture dishes (BD Falcon; Nippon Becton Dickinson) at a density of 9.0x10<sup>5</sup> cells/dish. After 24 h, the cells were treated with 100  $\mu$ M celecoxib in culture medium containing 2 or 10% FBS. Twenty-four hours later, culture media samples were collected. They were then centrifuged immediately at 500 x g for 5 min at 4°C to remove cells or debris, and the supernatants were harvested. The concentration of PGE<sub>2</sub> in the culture medium was measured using a PGE<sub>2</sub> enzyme immunoassay kit - Monoclonal (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's instructions. The absorbance at 405 nm was measured using a Benchmark Plus microplate reader (Bio-Rad Laboratories). The experiment was performed in triplicate.

**Flow cytometric cell cycle analysis.** AZACB cells were seeded at a density of 5.0x10<sup>5</sup> cells in 100-mm tissue culture dishes (BD Falcon; Nippon Becton Dickinson). After 24 h of exposure to selective COX-2 inhibitors (10, 25, 50 and 100  $\mu$ M meloxicam and etodolac, and 10, 25, 45, 50, 75 and 100  $\mu$ M celecoxib), AZACB cells were harvested and washed with PBS, resuspended in 70% ethanol in PBS, and frozen at -30°C overnight. Before analysis, the cells were incubated for 15 min in propidium iodide (PI)/RNase Staining Buffer (BD Pharmingen, San Diego, CA, USA) in the dark. The suspension was then filtered into a 5-ml polystyrene round-bottomed tube with a cell-strainer cap and was analyzed using FACSCanto (both from Becton-Dickinson, Franklin Lakes, NJ, USA). Data analyses were performed using FlowJo 7 (Tree Star, Inc., Ashland, OR, USA).

Table I. Real-time RT-PCR primer sequences.

Gene	Forward (5'→3')	Reverse (5'→3')
<i>GAPDH</i>	ATTCTATCCACGGCAAATCC	GGACTCCACAACATACTCAG
<i>p21</i>	CCTAATCTGCTCACCGGAAG	GGTGGCAAGCAGGGTATGTA
<i>p27</i>	CTCAGGCCAACTCAGAGGAC	TCTTAGGCGTCTGCTCCACT
<i>Bcl-2</i>	TGAACCGGCATCTGCACAC	GAGCAGCGCCTTCAGAGACA

RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Assessing changes in mitochondrial potential.** Mitochondrial permeability and membrane depolarization were measured using a MitoPT<sup>®</sup> tetramethylrhodamine ethyl ester (TMRE) assay kit (ImmunoChemistry Technologies, LLC, Bloomington, MN, USA) according to the manufacturer's instructions. AZACB cells were seeded into 100-mm dishes (BD Falcon; Nippon Becton Dickinson) at a density of  $3 \times 10^5$  cells/dish. After 24 h of treatment with selective COX-2 inhibitors, the cells were exposed to TMRE. The mitochondrial fluorescence intensity was measured using FACSCanto (488 nm excitation and 574 nm emission), and data were analyzed using FlowJo 7 (Tree Star, Inc.).

**Annexin V/PI staining and flow cytometry.** The different stages of apoptosis were analyzed using the ApoAlert<sup>®</sup> Annexin V-fluorescein isothiocyanate (FITC) Apoptosis kit (Clontech Laboratories, Mountain View, CA, USA) following the manufacturer's instructions. Both adherent and non-adherent cells were harvested using 0.25% of trypsin, and then centrifuged at 1,200 rpm for 5 min. The cell pellets were then washed and resuspended in binding buffer, and then incubated with Annexin V-FITC and PI for 15 min in the dark at room temperature. The samples were analyzed using FACSCanto, and the data were analyzed using FlowJo 7.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was isolated from AZACB cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and was reverse transcribed to cDNA using PrimeScript<sup>™</sup> RT kit (Takara Bio, Inc., Shiga, Japan) following the manufacturer's instructions as described previously (16,19-21). Real-time PCR was performed using SYBR Premix Ex Taq<sup>™</sup> II (Takara Bio, Inc.), an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Inc., Foster City, CA, USA) and the following conditions: 95°C for 30 sec, and 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was used as an internal control. The primer sequences used to amplify *p21*, *p27*, *Bcl-2*, and *GAPDH* are shown in Table I. The primers for *Bcl-2* were purchased from Takara Bio, Inc., and the other primers were obtained from Operon Biotechnology (Tokyo, Japan). All samples were amplified in triplicate in each experiment. The relative expression levels of mRNA were calculated using the comparative threshold cycle (Ct) method.

**Measuring caspase-3/7, -8, and -9 activity.** Caspase-3/7, -8, and -9 activity was analyzed using Caspase-Glo<sup>®</sup> 3/7, 8 and 9

assay kits (Promega Corp., Madison, WI, USA), respectively, following the manufacturer's instructions. Cells were cultured in white opaque tissue culture plates (BD Falcon; Nippon Becton Dickinson) at a density of  $2.5 \times 10^3$  cells/well. Twenty-four hours after drug treatment (100  $\mu$ M meloxicam and etodolac, or 10, 25, 40, 50, 75, and 100  $\mu$ M celecoxib), the fluorescence was measured every 30 min for up to 180 min using the LB 960 Microplate Luminometer Centro (Berthold Japan K.K., Tokyo, Japan). All samples were measured in triplicate in each experiment.

**Statistical analysis.** Data are presented as means  $\pm$  SD. Statistical analyses were performed using the Bonferroni test or Mann-Whitney test to identify significant differences between the selective COX-2 inhibitor-treated cells and control cells.  $P < 0.05$  was considered statistically significant.

## Results

**Celecoxib inhibits the proliferation of AZACB cells.** We reported previously that AZACB cells expressed lower levels of COX-2 protein than CF33 cells (18). In the current study, we confirmed that AZACB cells expressed the lowest levels of COX-2 among various canine mammary tumor cell lines, including CF33 and CF41.MG cells (Fig. 1A). Next, we assessed the effect of the selective COX-2 inhibitors meloxicam, etodolac, and celecoxib on cell viability to determine whether they inhibited the proliferation of AZACB cells. We measured cell proliferation using WST-8 assays 24 h after treatment with the selective COX-2 inhibitors. There was no difference in proliferation between the parent and control cells. As shown in Fig. 1D, 75 and 100  $\mu$ M celecoxib significantly induced growth arrest 24 h after treatment compared with control cells; however, meloxicam and etodolac had no significant effect (Fig. 1B and C). These results suggest that celecoxib markedly inhibited the proliferation of AZACB cells.

**Celecoxib inhibits the proliferation of AZACB cells mainly via COX-2-independent mechanisms.** Numerous reports have suggested that NSAIDs exert antitumor effects on human cancer cells via COX-2-independent mechanisms (24). Next, we evaluated whether celecoxib inhibited cell proliferation in a COX-2-dependent or -independent manner by examining the effect of DMC on the proliferation of canine mammary tumor cells. DMC is a structural isomer of celecoxib that lacks COX-2-inhibitory activity (25). As shown in Fig. 2A, DMC

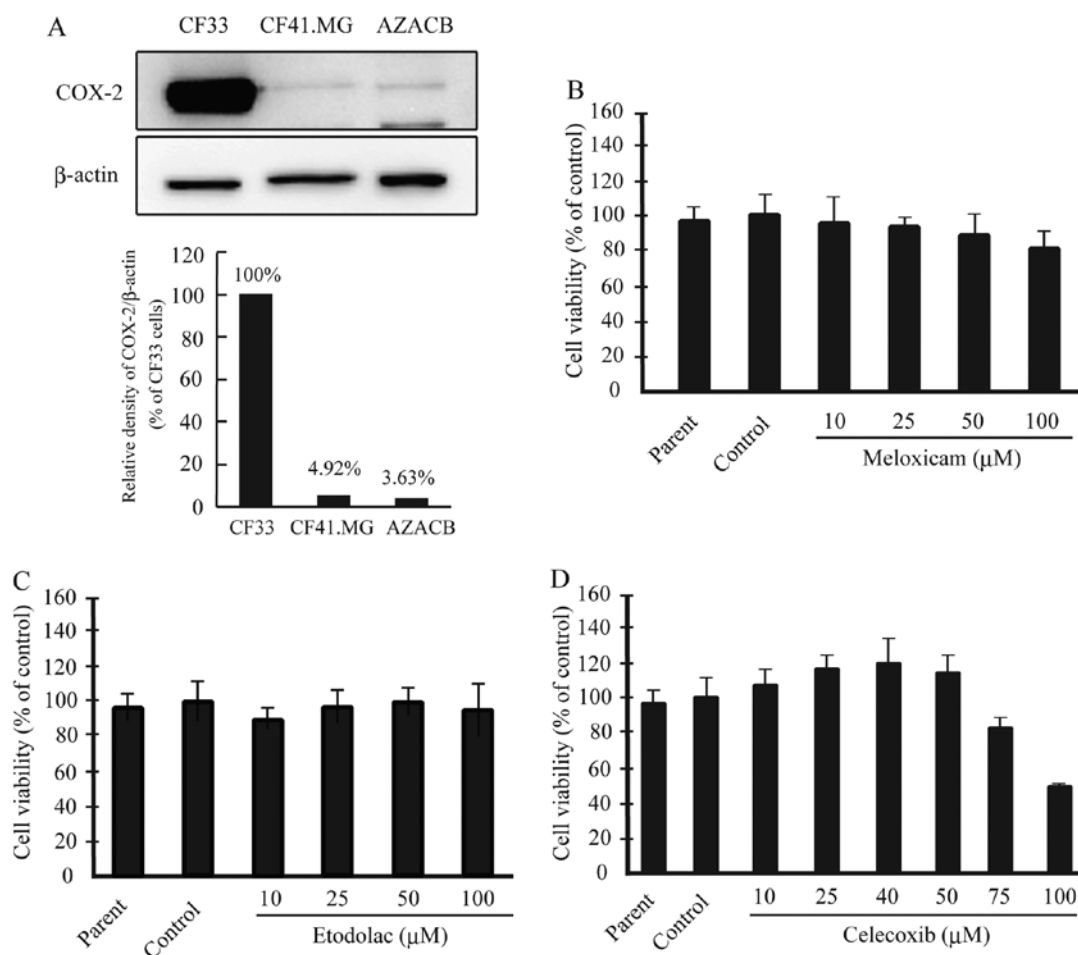


Figure 1. Celecoxib inhibits the proliferation of AZACB cells. (A) Western blotting to assess the levels of cyclooxygenase-2 (COX-2) in CF33, CF41.MG, and AZACB cells. (B-D) The effects of selective COX-2 inhibitors on cell proliferation were analyzed using WST-8 assays 24 h after treatment. Data are presented as means  $\pm$  SD (n=5). The viability of AZACB cells treated with (B) meloxicam, (C) etodolac and (D) celecoxib. Control cells were treated with 0.1% DMSO, and parent cells were untreated.

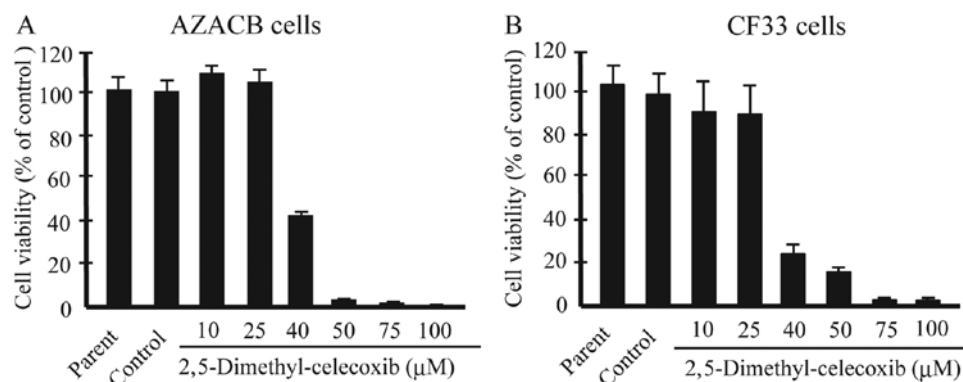


Figure 2. Celecoxib inhibits the proliferation of AZACB cells via cyclooxygenase-2 (COX-2)-independent mechanisms. (A and B) The effects of 2,5-dimethyl-celecoxib (DMC) on cell proliferation were analyzed using WST-8 assays 24 h after treatment. The data are presented as means  $\pm$  SD (n=5).

inhibited the proliferation of AZACB and CF33 cells, which express low and high levels of COX-2, respectively (Fig. 2B). This suggests that celecoxib inhibited the proliferation of canine mammary tumor cells via COX-2-independent mechanisms.

We demonstrated previously that celecoxib downregulated the expression of COX-2 in CF33 cells (16). As shown in Fig. 3A and B, COX-2 protein expression was reduced only

in AZACB cells treated with 100  $\mu$ M celecoxib. It is well known that COX-2 catalyzes the production of PGs such as PGE<sub>2</sub>. However, despite the reduced expression of COX-2, no significant changes in PGE<sub>2</sub> production were observed in celecoxib-treated AZACB cells compared with control cells (Fig. 3C). These results suggest that celecoxib-induced growth inhibition in AZACB cells is mediated mainly

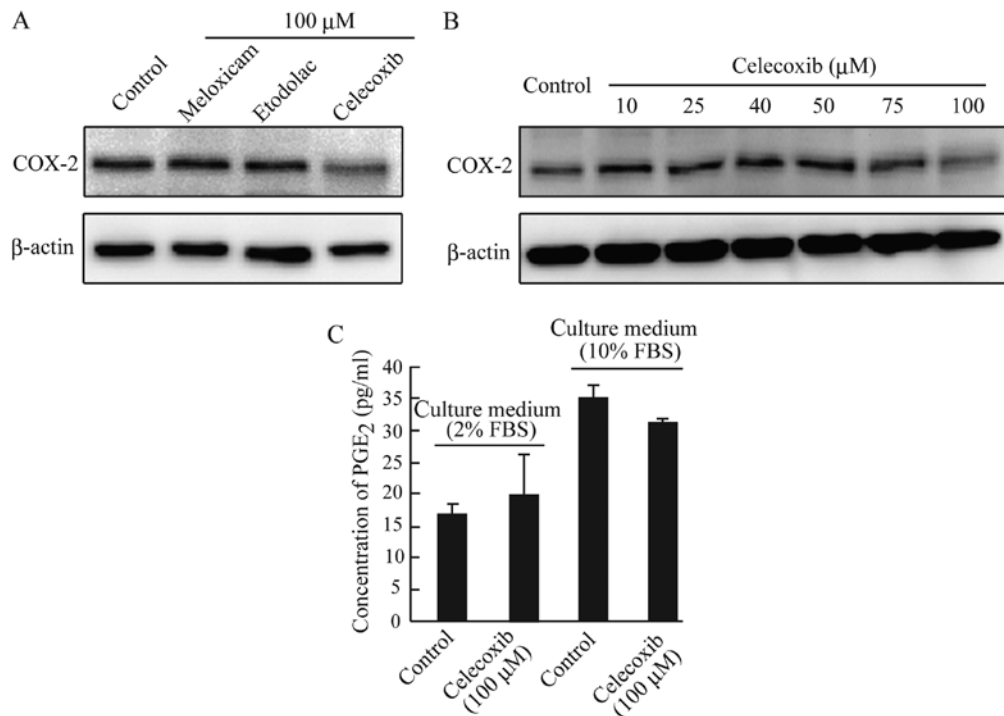


Figure 3. Celecoxib downregulates cyclooxygenase-2 (COX-2) expression without affecting prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels in AZACB cells. (A) Western blotting of COX-2 levels in AZACB cells treated with selective COX-2 inhibitors for 24 h. (B) Western blotting of COX-2 levels in AZACB cells treated with the indicated doses of celecoxib. (C) Enzyme immunoassay for PGE<sub>2</sub> secretion into culture medium containing 2 or 10% fetal bovine serum (FBS) in AZACB cells treated with celecoxib for 24 h. The experiment was performed in triplicate, and the data are presented as means  $\pm$  SD.

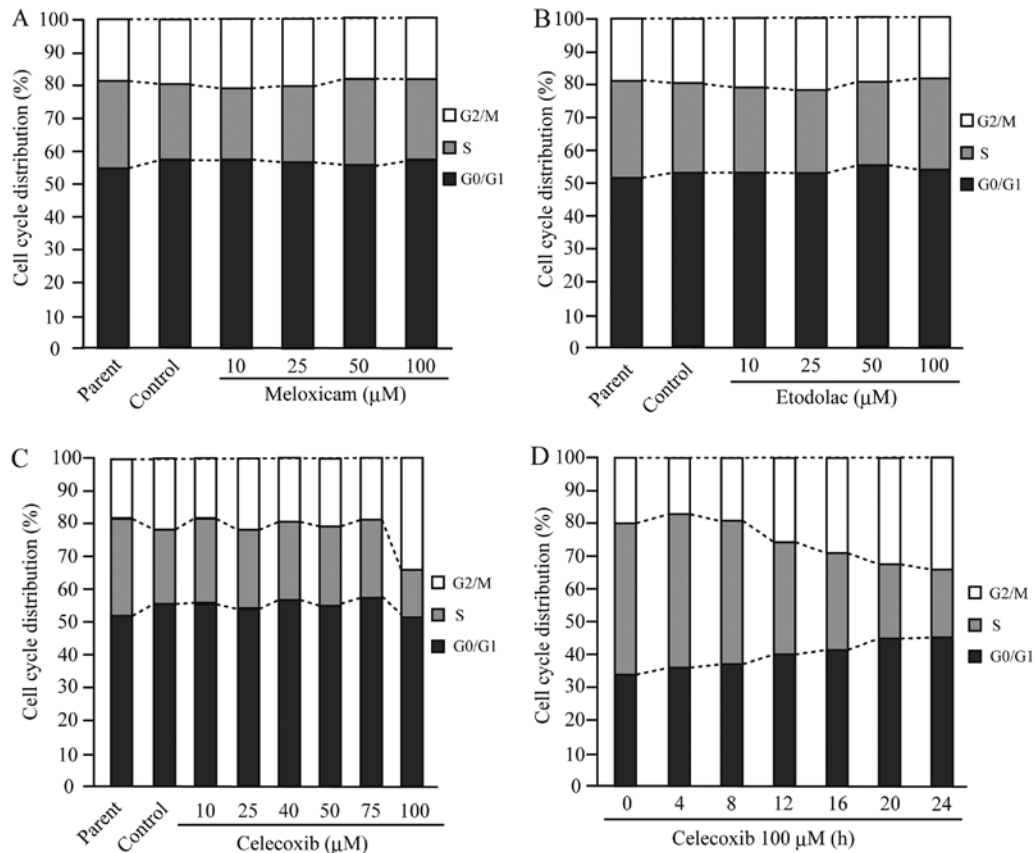


Figure 4. Celecoxib decreases the number of cells in S phase and increases the number of those in G2/M arrest. (A-D) The effects of selective cyclooxygenase-2 (COX-2) inhibitors on the distribution of cells in the cycle stage were analyzed using flow cytometry of propidium iodide (PI)-stained cells. (A-C) The percentage of cells treated with 10-100  $\mu$ M meloxicam, etodolac and celecoxib, respectively, distributed in G0/G1, S, and G2/M phases of the cell cycle. (D) The distribution of AZACB cells in each stage of the cell cycle after treatment with 100  $\mu$ M celecoxib for the indicated times. Control cells were treated with 0.1% DMSO, and parent cells were not treated. A total of 20,000 cells were analyzed in each experiment.

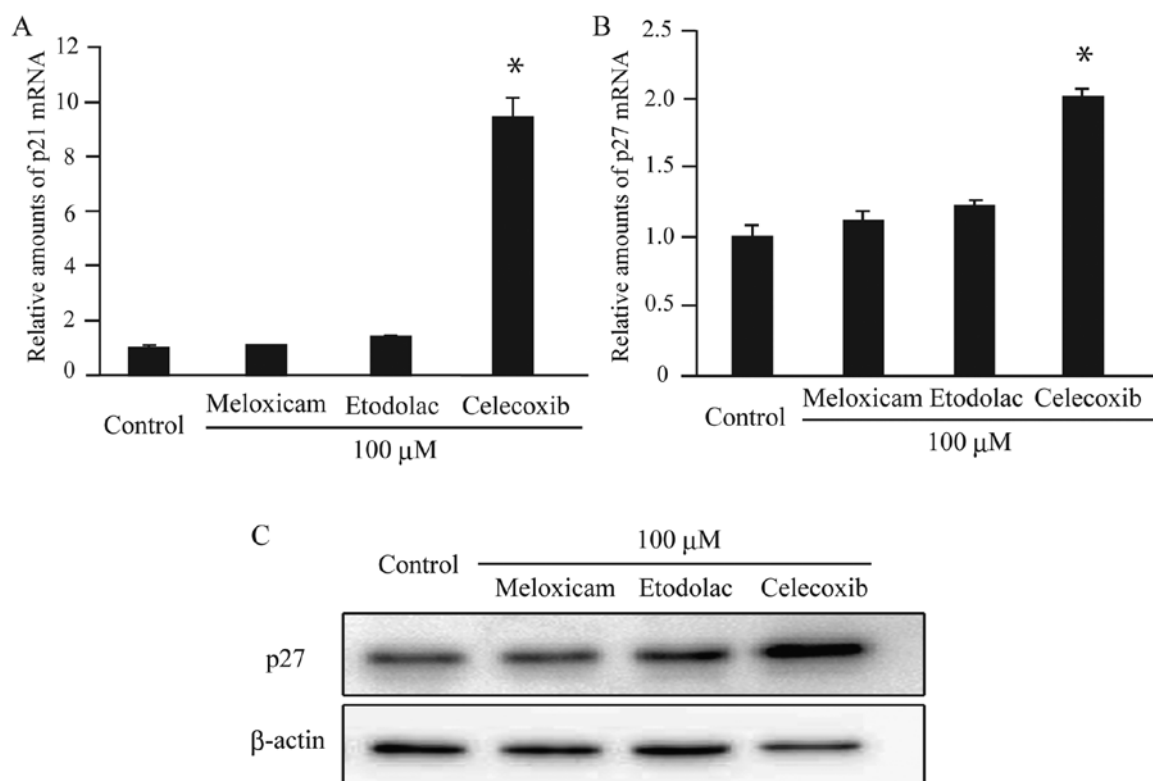


Figure 5. Celecoxib increases the levels of the cyclin-dependent kinase inhibitors (CDKIs) *p21* and *p27* in AZACB cells. (A-B) Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *p21* and *p27* mRNA levels in AZACB cells treated with selective cyclooxygenase-2 (COX-2) inhibitors. The data are presented as means  $\pm$  SD ( $n=3$ ). \* $P<0.01$  compared with control cells. (C) Western blotting shows the expression of *p27* protein in AZACB cells treated with selective COX-2 inhibitors.

by COX-2-independent mechanisms. Furthermore, the unchanged PGE<sub>2</sub> secretion after celecoxib treatment might be caused by low levels of COX-2 expression compared with other canine mammary tumor cells. These results suggest that celecoxib might affect the expression and/or activation of proteins such as NF- $\kappa$ B, which regulates COX-2 expression (24,26-29).

*Celecoxib decreased the number of cells in S phase and increased G2/M arrest by upregulating p21 and p27.* We demonstrated previously that celecoxib treatment reduced the number of CF33 cells in S phase and increased those in G0/G1 (18). In addition, meloxicam and etodolac slightly induced G0/G1 arrest (18). As shown in Fig. 4A and B, there was no significant change in the cell cycle distribution patterns in AZACB cells treated with etodolac and meloxicam. However, treatment with 100  $\mu$ M celecoxib markedly induced G2/M arrest and decreased the number of cells in S phase (Fig. 4C). Furthermore, this effect occurred after 12 h of treatment (Fig. 4D). To confirm that celecoxib induced cell cycle arrest at the G2/M phase, we next evaluated the expression of the cyclin-dependent kinase inhibitors (CDKIs) *p21* and *p27*. There were no changes in the expression of *p21* and *p27* in meloxicam- or etodolac-treated cells (Fig. 5A-C). In contrast, treatment with celecoxib increased the levels of both *p21* and *p27* in AZACB cells (Fig. 5A-C). Therefore, these data suggest that celecoxib induced G2/M arrest and reduced the number of AZACB cells in S phase by increasing the expression of CDKIs, including *p21* and *p27*.

*Treatment with 100  $\mu$ M celecoxib markedly induced AZACB cell apoptosis.* We recently reported that celecoxib markedly inhibited the proliferation of CF33 canine mammary tumor cells by inducing apoptosis (18). Therefore, we next assessed the effects of meloxicam, etodolac, and celecoxib on apoptosis in AZACB cells. As shown in Fig. 6A and B, there were no changes in apoptosis in meloxicam- or etodolac-treated AZACB cells. However, as expected, treating cells with 100  $\mu$ M celecoxib induced apoptosis (Fig. 6C); these effects were time-dependent (Fig. 6D). To confirm these observations, we next analyzed apoptosis using Annexin V/PI double staining. Data revealed that treatment with 100  $\mu$ M celecoxib for 24 h induced both early and late apoptosis (Fig. 6E and F). As expected, treating AZACB cells with 100  $\mu$ M meloxicam or etodolac had no effect on either early or late apoptosis (Fig. 6E and F).

The imbalance between pro-apoptotic (Bax, Bim, and Bak) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) leads to apoptosis by stimulating mitochondrial outer membrane permeabilization (MOMP) (30). Accordingly, we assessed changes in the expression of apoptotic proteins in celecoxib-treated AZACB cells. Celecoxib-treated AZACB cells exhibited elevated Bax and Bim expression, and reduced Bcl-2 expression (Fig. 7A-D). However, levels of Bax were only slightly elevated in celecoxib-treated AZACB cells (Fig. 7A and C). These changes were induced by treatment with  $\geq 75$   $\mu$ M celecoxib (Fig. 7C and D). It is known that MOMP leads to a decrease in mitochondrial membrane potential (31). Therefore, we analyzed changes in mitochondrial membrane

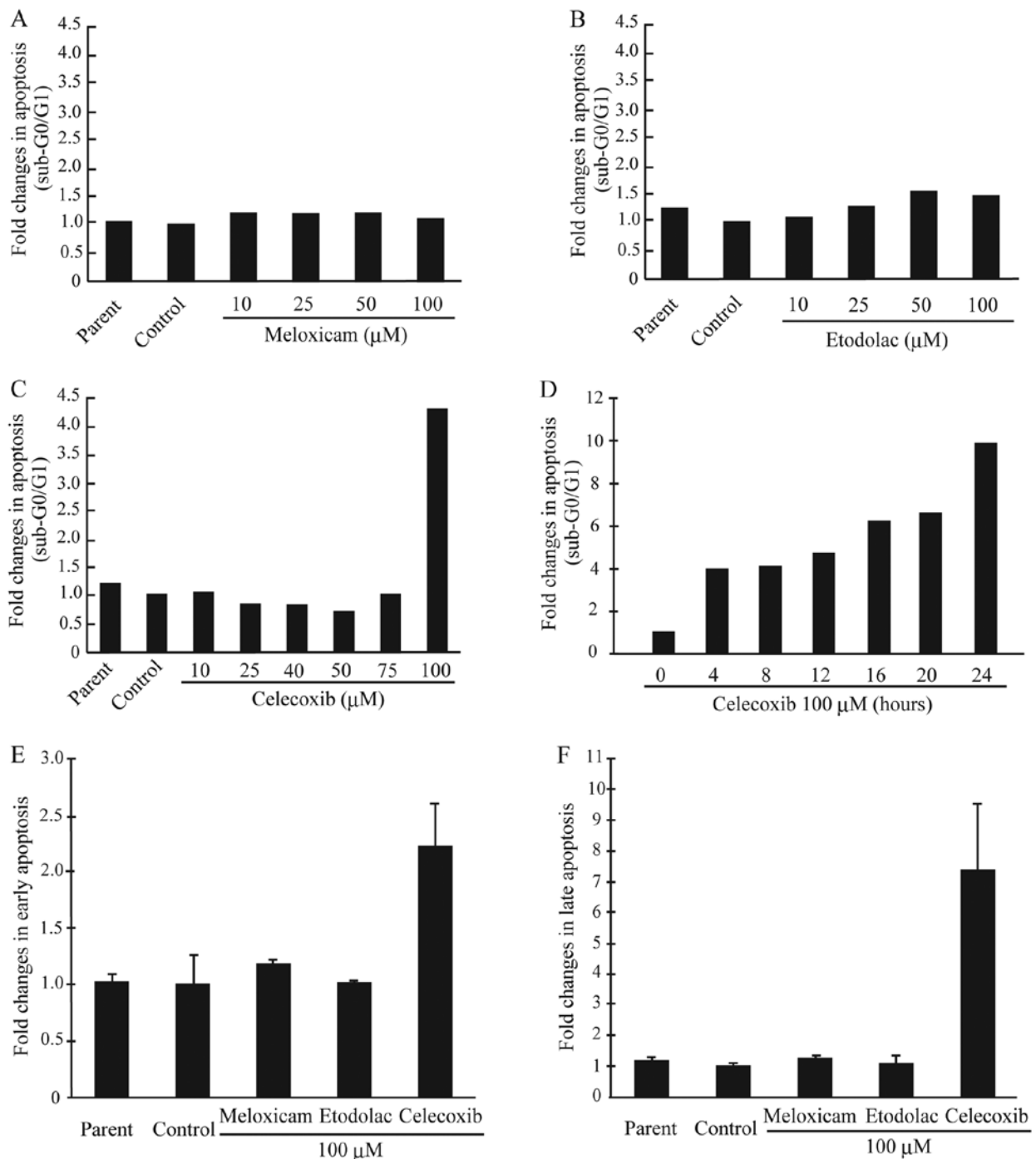


Figure 6. Treatment with 100  $\mu$ M celecoxib induces apoptosis in AZACB cells. (A-C) The apoptosis induced by selective cyclooxygenase-2 (COX-2) inhibitors was analyzed by assessing propidium iodide (PI)-stained cells by FACS analysis. The data show the cells in the sub-G0/G1 peak 24 h after treatment with 0-100  $\mu$ M of the selective COX-2 inhibitors meloxicam, etodolac, and celecoxib, respectively, for 24 h. (D) The sub-G0/G1 peak of AZACB cells treated with 100  $\mu$ M celecoxib for the indicated times. (E-F) The apoptotic effects of 100  $\mu$ M celecoxib in AZACB cells were confirmed using flow cytometry to analyze Annexin V-fluorescein isothiocyanate (FITC) and PI double-stained cells. The data are presented as means  $\pm$  SD. (E) The number of early apoptotic cells (Annexin V-positive, PI-negative). (F) The number of late apoptotic cells (Annexin V-positive, PI-positive). Control cells were treated with 0.1% DMSO, and parent cells were not treated. A total of 20,000 cells were analyzed in each experiment.

potential using flow cytometric analysis of TMRE-stained cells to directly measure whether celecoxib induced MOMP. Our results showed that treating cells with 100  $\mu$ M celecoxib, but not meloxicam and etodolac, decreased mitochondrial membrane potential (Fig. 7E). Moreover, the decrease in mitochondrial membrane potential was observed only in AZACB cells treated with 100  $\mu$ M celecoxib, and not lower

doses (Fig. 7F). These findings confirm that celecoxib induces apoptosis in AZACB cells, which express low levels of COX-2.

*Celecoxib induces apoptosis in AZACB cells by activating both the intrinsic and extrinsic apoptotic pathways.* To further clarify the effect of selective COX-2 inhibitors on apoptosis, we analyzed the caspase-3/7 activity. Only celecoxib induced

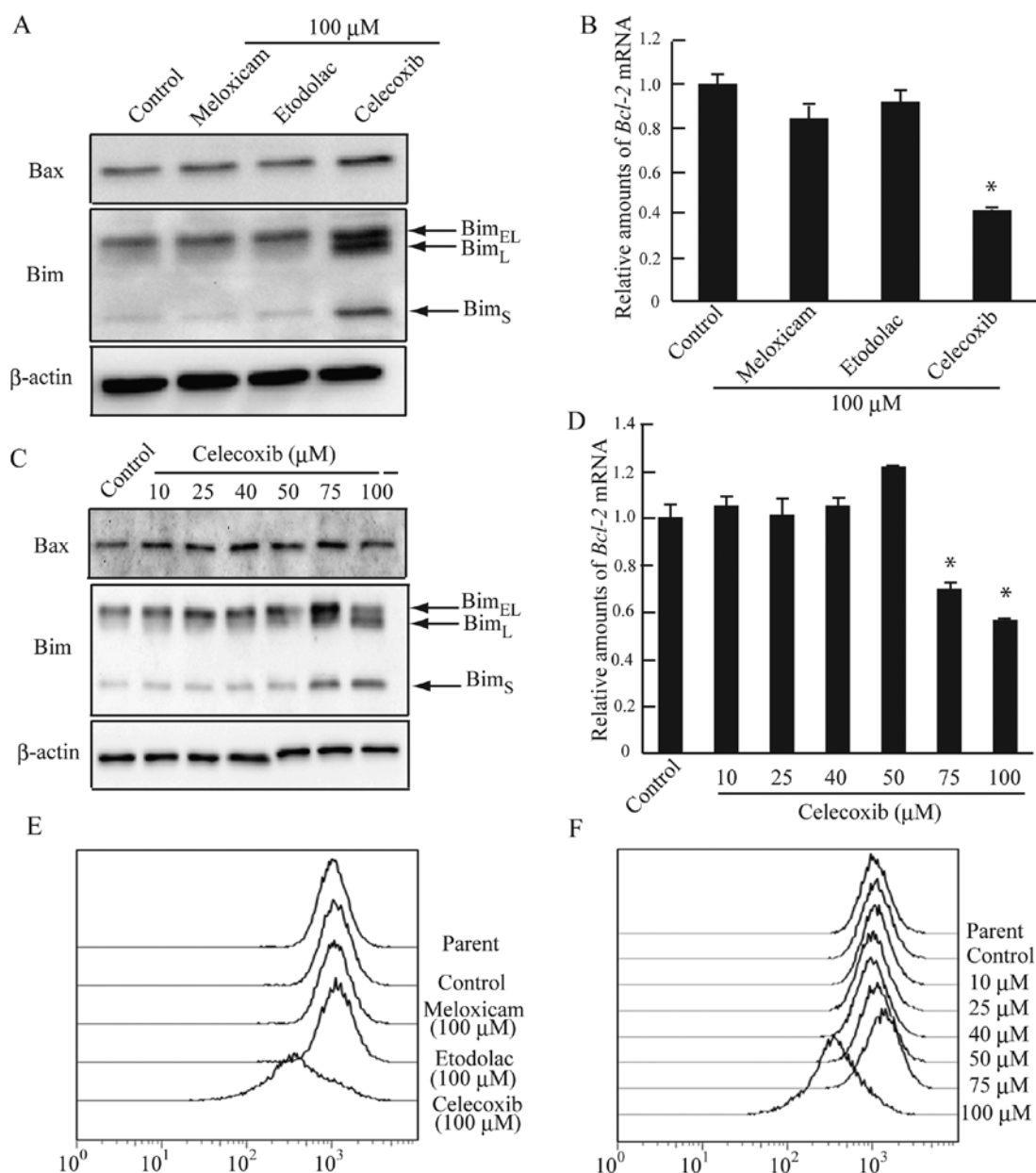


Figure 7. Celecoxib alters the expression of *Bcl-2* family members, which decreased mitochondrial membrane potential. (A) Western blotting of the expression of the pro-apoptotic proteins Bax and Bim in AZACB cells treated with selective cyclooxygenase-2 (COX-2) inhibitors for 24 h. (B) Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *Bcl-2* mRNA levels in AZACB cells treated with selective COX-2 inhibitors. The data are presented as means  $\pm$  SD (n=3). \*P<0.01 compared with control cells. (C) Western blotting of the expression of the pro-apoptotic proteins Bax and Bim in AZACB cells treated with the indicated doses of celecoxib. (D) Real-time RT-PCR analysis of *Bcl-2* mRNA levels in AZACB cells treated with the indicated doses of celecoxib. The data are presented as means  $\pm$  SD (n=3). \*P<0.01 compared with control cells. (E-F) The altered mitochondrial membrane depolarization caused by selective COX-2 inhibitors was confirmed by analyzing tetramethylrhodamine ethyl ester (TMRE)-stained cells using flow cytometry. A total of 20,000 cells were analyzed in each experiment. The control cells were treated with 0.1% DMSO, and the parent cells were not treated.

the activation of caspase-3/7 (Fig. 8A), and this effect required treatment with a dose of 100  $\mu$ M celecoxib (Fig. 8B). Caspase-dependent apoptosis is divided into the intrinsic and extrinsic pathways, which are induced by the activation of initiator caspase-8 or -9, respectively (32-34). The subsequent activation of the effector caspase-3 and -7 then leads to apoptosis (35). To determine whether celecoxib induced apoptosis via the intrinsic or extrinsic apoptotic pathway, we measured the activity of caspase-8 and -9 in AZACB cells treated with selective COX-2 inhibitors. As shown in Fig. 8C and D, celecoxib induced the activation of both caspase-8 and -9 in AZACB cells. Celecoxib also induced the cleavage of

Bid to truncated Bid (Fig. 8E). These data suggest that celecoxib-induced apoptosis is mediated by both the intrinsic and extrinsic apoptotic pathways.

To confirm the activation of these apoptotic pathways in celecoxib-treated cells, we next assessed the effect of caspase-8 and -9 inhibitors on celecoxib-induced apoptosis in AZACB cells. The caspase-8 inhibitor Z-IETD-FMK completely inhibited celecoxib-induced caspase-8 activation (Fig. 9A). However, it did not completely block celecoxib-induced apoptosis (Fig. 9C). Similarly, the caspase-9 inhibitor Z-LEHD-FMK significantly inhibited celecoxib-induced caspase-9 activation in AZACB cells (Fig. 9B).



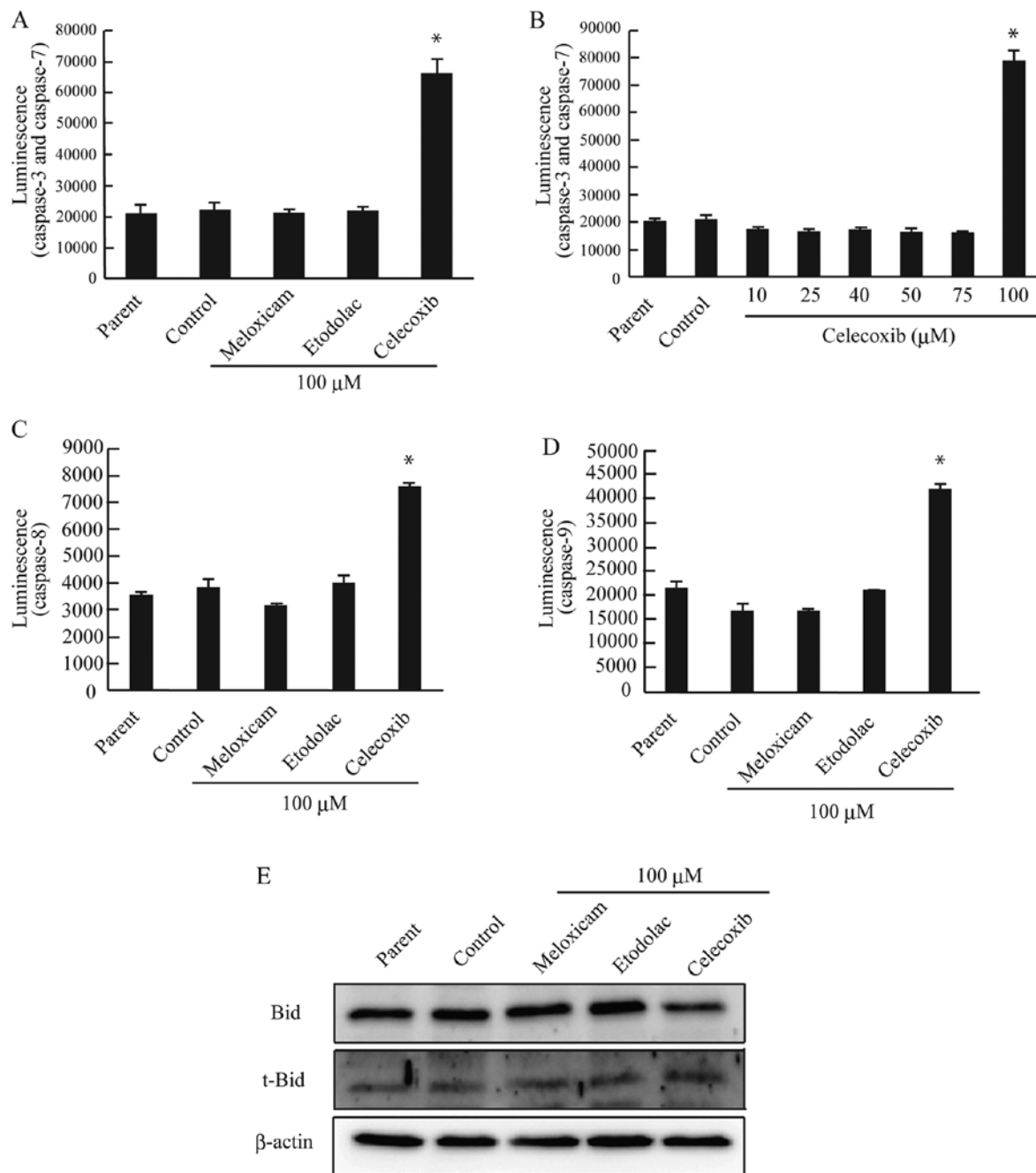


Figure 8. Celecoxib activates both caspase-8 and -9, which subsequently induced apoptosis by increasing caspase-3/7 activity in AZACB cells. (A) Caspase-3/7 activity after treatment with 100  $\mu$ M meloxicam, etodolac, and celecoxib for 24 h was measured using Caspase-Glo<sup>®</sup> 3/7 assays. (B) Caspase-3/7 activity after treatment with the indicated doses of celecoxib for 24 h was measured using Caspase-Glo<sup>®</sup> 3/7 assays. (C) Caspase-8 activity after treatment with 100  $\mu$ M meloxicam, etodolac, and celecoxib for 24 h was measured using Caspase-Glo<sup>®</sup> 8 assays. (D) Caspase-9 activity after treatment with 100  $\mu$ M meloxicam, etodolac, and celecoxib for 24 h was measured using Caspase-Glo<sup>®</sup> 9 assays. (E) Celecoxib induced the cleavage of Bid to truncated Bid. The control cells were treated with 0.1% DMSO, and the parent cells were not treated. The data are presented as means  $\pm$  SD (n=3). \*P<0.01 compared with control cells.

However, it did not completely inhibit celecoxib-induced apoptosis (Fig. 9D). These results strongly support the notion that celecoxib-induced apoptosis is mediated by both the intrinsic and extrinsic apoptotic pathways in AZACB cells.

## Discussion

In humans, the regular intake of NSAIDs such as aspirin and selective COX-2 inhibitors is associated with a decreased risk of cancer incidence, distant recurrence, and cancer-related

deaths in various cancers, including breast and colon cancers (36,37). However, it largely remains unclear whether NSAIDs might be useful chemotherapy or chemopreventative agents in canine mammary tumors. Our findings revealed that celecoxib inhibited cell proliferation by decreasing the number of cells in S phase and increasing G2/M arrest by stimulating the expression of the CDKIs p21 and p27 in AZACB cells. Furthermore, our findings suggest that celecoxib might exert antitumor effects mainly via COX-2-independent mechanisms in canine mammary tumor cells. In addition, celecoxib might

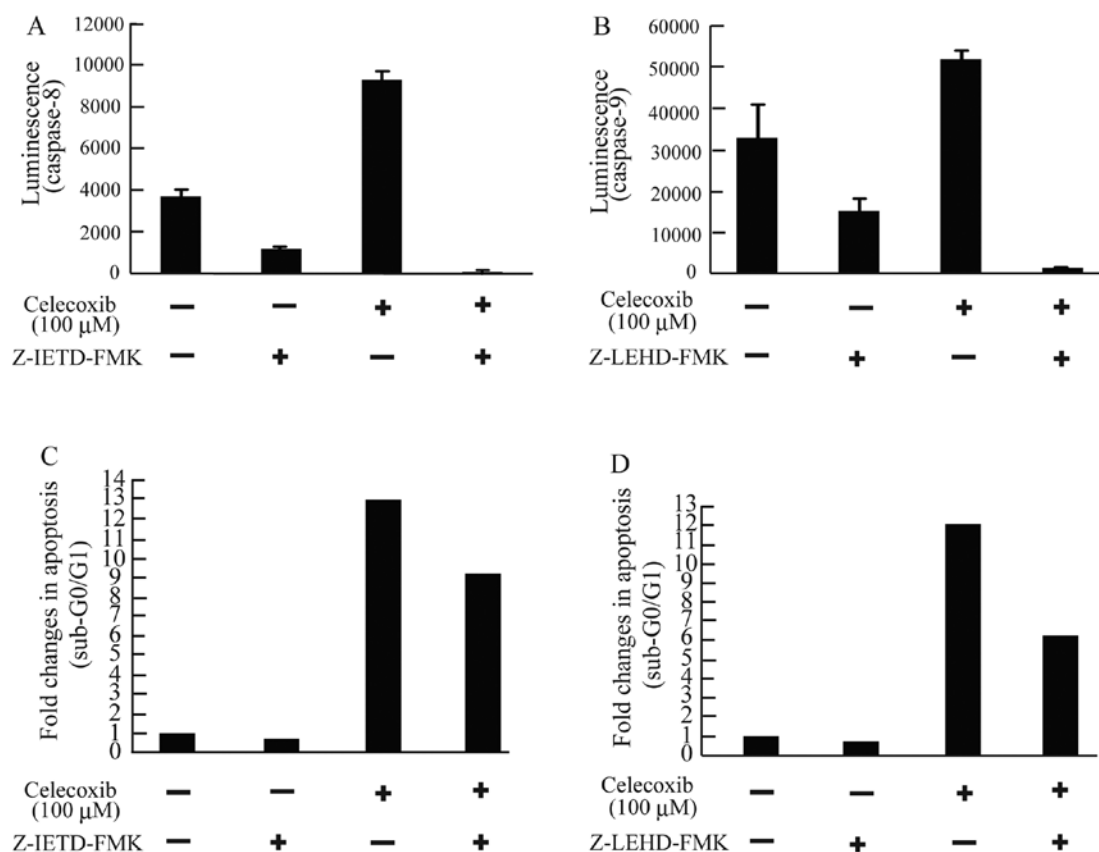


Figure 9. Celecoxib induces apoptosis via both the intrinsic and extrinsic apoptotic pathways in AZACB cells. (A-B) To confirm the inhibitory effects of caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) on caspase-8 and -9 activities, we analyzed the respective caspase activities using Caspase-Glo<sup>®</sup> 8 and 9 assays, respectively. Cells were treated with each inhibitor at a final concentration of 20  $\mu$ M. Control cells were treated with DMSO only. The data are presented as means  $\pm$  SD (n=3). (C-D) To evaluate whether celecoxib-induced apoptosis was dependent on the intrinsic or extrinsic apoptotic pathway, AZACB cells treated with caspase inhibitors and/or celecoxib for 24 h were analyzed using FACS analysis. A total of 20,000 cells were analyzed in each experiment. Control cells were only treated with DMSO. Cells were treated with each inhibitor at a final concentration of 20  $\mu$ M.

induce apoptosis by activating both the intrinsic and extrinsic apoptotic pathways in AZACB cells. We demonstrate, for the first time, that celecoxib shows potential be a useful chemotherapy agent in canine mammary tumor cells, regardless of COX-2 expression levels.

Several studies have reported that the aberrant overexpression of COX-2 is observed in human cancers such as breast cancer, prostate cancer, lung cancer, and colorectal adenomas and carcinomas (38). In addition, elevated COX-2 levels were associated with unfavorable outcome, lymph node metastasis, and distant metastasis (39-41). Therefore, various studies have suggested that selective COX-2 inhibitors might be useful chemopreventative and chemotherapeutic agents in human breast cancer. Similar to human breast cancer, some reports have identified correlations between COX-2 expression and angiogenesis, poor prognosis, and the development of distant metastases in canine mammary tumors (17,42,43). Consistent with this, we demonstrated previously that selective COX-2 inhibitors, particularly celecoxib, had powerful antitumor activity in CF33 cells that was mediated by the induction of apoptosis (18). Furthermore, the current study suggests that celecoxib exhibited antitumor effects in canine mammary tumor cells regardless of COX-2 expression. These results strongly support the hypothesis that celecoxib might be a useful chemotherapeutic agent in canine mammary tumor

cells. Furthermore, celecoxib might be a potent adjunctive therapeutic agent for intractable canine mammary tumors.

Apoptosis (programmed cell death), plays a key role in the development and regulation of tissue homeostasis. Apoptosis can be triggered by at least two major pathways: the intrinsic (mitochondrial) and the extrinsic (death-receptor) pathway. In the extrinsic pathway, caspase-8 is activated after the interaction of death receptors, including CD95, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-R1 and -R2, as well as TNF-receptor-1, with their cognate ligands CD95, TRAIL, and TNF, respectively (32,34). In contrast, in the intrinsic pathway, caspase-9 is activated by MOMP followed by an imbalance between pro- and anti-apoptotic proteins (30,32-34). These distinct pathways then converge to activate the effector caspase-3 and -7 (30). However, it remains controversial whether celecoxib-induced apoptosis is mediated by the intrinsic or extrinsic pathway. Celecoxib-induced apoptosis occurred via the intrinsic pathway and a Bak-dependent, Bcl-2-independent pathway in Jurkat T-lymphoma cells (44,45). In contrast, Liu *et al* reported that celecoxib-induced apoptosis in human non-small cell lung cancer cell lines was mediated by activation of the extrinsic pathway following increased TRAIL-R2 expression, enhanced TRAIL-induced apoptosis, and the downregula-

tion of cellular FADD-like interleukin-1  $\beta$ -converting enzyme-inhibitory protein (46,47).

The current study suggests that celecoxib activated both the intrinsic and extrinsic pathways in AZACB cells, which was mediated by the activation of the initiator caspase-8 and -9. In the intrinsic pathway, our data suggest that an imbalance between pro-apoptotic (Bax and Bim) and anti-apoptotic (Bcl-2) proteins leads to breakdown of mitochondrial membrane potential, which sequentially activates caspase-9 and -3/7. In the extrinsic pathway, celecoxib activates caspase-3/7 via -8 in AZACB cells. Interestingly, Bid plays a key role in crosstalk between the intrinsic and extrinsic apoptotic pathways (34). Therefore, the findings of the current study support the notion that the cleavage of Bid by active caspase-8 might induce mitochondrial depolarization in celecoxib-treated AZACB cells. In addition, specific inhibitors of caspase-8 and -9 could not completely block celecoxib-induced apoptosis in AZACB cells. The present study showed, for the first time, that activation of both the intrinsic and extrinsic apoptotic pathways play a critical role in celecoxib-induced apoptosis in canine mammary tumors. A recent study reported that Bim, a pro-apoptotic Bcl-2 homology domain 3-only protein, is a critical mediator of anoikis in MCF10A cells (48). The current study demonstrated that a high dose of celecoxib significantly upregulated Bim protein expression in AZACB cells. These results suggest that celecoxib induced anoikis in AZACB cells.

The interaction of COX-2-derived PGs (e.g., PGE<sub>2</sub>) with their receptors (e.g., EP1, 2, 3, and 4) induces apoptosis resistance, cell proliferation, angiogenesis, invasion, and metastasis (49-54). However, it was revealed previously that NSAIDs exert antitumor activities in cancer cells via both COX-2-dependent and -independent mechanisms (41). For COX-2-independent mechanisms, it was proposed that NSAIDs affect gene expression patterns or the activation of various molecules such as NF- $\kappa$ B, pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1)/Akt, p21, and peroxisome proliferator-activated receptors (24). In particular, NF- $\kappa$ B plays a critical role in cell survival and regulates the expression of various genes in cancer cells. NF- $\kappa$ B is generally localized in the cytoplasm in its inactive form bound to its inhibitor protein I $\kappa$ B $\alpha$ . The phosphorylation and subsequent proteasomal degradation of I $\kappa$ B $\alpha$  leads to the nuclear translocation of NF- $\kappa$ B (55). Furthermore, COX-2 expression is regulated by NF- $\kappa$ B in various cells (26-29). Therefore, it is possible that NF- $\kappa$ B might be a key molecule in both the celecoxib-induced downregulation of COX-2 and COX-2-independent antitumor effects in canine mammary tumor cells. Our data also demonstrated that celecoxib enhanced the expression of both p21 and p27. Therefore, our observations suggest that both p21 and p27 might play critical roles in celecoxib-induced COX-2-independent antitumor mechanisms in canine mammary tumors.

Recently, Seo *et al* reported that celecoxib exerted antitumor effects in both COX-2-expressing and -non-expressing canine melanoma cells (56). Consistent with this, the current study demonstrated that a high dose of celecoxib induced growth arrest and apoptosis in canine mammary tumor cells, regardless of COX-2 expression. Taken together, these data suggest that celecoxib might act via both COX-2-dependent and -independent mechanisms in various canine cancers. Accordingly,

selective COX-2 inhibitors might cause more favorable therapeutic responses in various canine cancers compared with human or other cancers.

In conclusion, our findings support the hypothesis that celecoxib might be a viable chemotherapy or chemopreventative agent in canine mammary tumors, regardless of COX-2 expression. In particular, celecoxib exerts antitumor activity via COX-2-independent mechanisms in canine mammary tumors. In the future, it might be possible to use a combination of celecoxib and other antitumor drugs to treat canine mammary tumors, regardless of their COX-2 expression status.

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