

# COX-2 independent induction of apoptosis by etodolac in leukemia cells *in vitro* and growth inhibition of leukemia cells *in vivo*

Research Article

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**Key Words:** COX-2, apoptosis, leukemia cells, growth inhibition, PGE<sub>2</sub>, NSAIDs

**Abbreviations:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (MTT); American Type Culture Collection, (ATCC); B-chronic lymphocytic leukemia, (CLL); cellular IAP-1, (cIAP-1); Cyclooxygenase-2, (COX-2); dimethyl sulfoxide, (DMSO); fetal calf serum, (FCS); inhibitor of apoptosis, (IAP); mouse monoclonal anti-caspase-3, (CPP32); multiple myeloma, (MM); Non-steroidal anti-inflammatory drugs, (NSAIDs); propidium iodide, (PI); prostaglandin, (PG); Tris-buffered saline Tween, (TBS-T); X-linked IAP, (XIAP);

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## Summary

Cyclooxygenase-2 (COX-2) has been reported to regulate apoptosis and influence the growth of malignancies. In this study, we demonstrated that etodolac, a COX-2 inhibitor, inhibited proliferation and induced apoptosis in leukemia K562, NB4, U937, HL60, and CEM cells via a COX-2 independent pathway. Etodolac induced apoptosis in a dose-dependent manner, which was associated with i) down-regulation of anti-apoptotic bcl-2, ii) activation of caspase -9, -7 and -3, iii) down-regulation of caspase inhibitors, c-IAP-1 and survivin, and iv) breakdown of the mitochondrial membrane potential. *In vivo*, etodolac also reduced the growth of K562 cells. Moreover, we found that 100 μM R- etodolac, S- etodolac, and the combination of R- and S- etodolac slightly inhibited the proliferation of leukemia cells, while 100 μM etodolac significantly inhibited the proliferation of leukemia cells. In conclusion, our findings further indicate that etodolac induce apoptosis in leukemia cells *in vitro* and inhibited tumor growth in a K562 nude mouse xenograft.

## I. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to exert anti-proliferative and pro-apoptotic effects on various cancer cell lines (Thun et al, 1991; Sun et al, 2002) and animal models of a variety of cancers, particularly colon cancer (Oshima et al, 1996; Kawamori et al, 1998). Cyclooxygenase (COX), a key enzyme was required for prostaglandin (PG) synthesis (Shattuck-Brandt et al, 2000). There are two different isoforms, that is, COX-1 is expressed constitutively in most tissues, whereas COX-2 is inducible through many pathological processes such as inflammation and in bearing cancers (Kujubu et al, 1991; Yamazaki et al,

2002). COX-2 overexpression has been reported in cancers of the colon (Piazza et al, 1997; Yamazaki et al, 2002), pancreas (Molina et al, 1999), breast (Half et al, 2002), lung (Hida et al, 1998), and mucous membrane of the head and neck (Wilson et al, 1998; Liu et al, 2001). The anti-proliferative and pro-apoptotic effects of selective COX-2 inhibitors have been reported recently for various cancers (Soslow et al, 2000; Nakanishi et al, 2001; Sun et al, 2002). Therefore, COX-2 might be a molecular target for cancer therapy. In the molecular mechanisms of COX-2 inhibitors, the pro-apoptotic effects of these might be exerted through down-regulation of anti-apoptotic molecules induced by COX-2. However, it was shown that some COX-2 inhibitors such as celecoxib induced

apoptosis in tumor cells which did not express the COX-2 enzyme, and COX-2 was not required as the effects of COX-2 inhibitors on induction of apoptosis.

In a family of proteins regulating apoptosis, bcl-2 inactivates pro-apoptotic molecules such as bax, bak, Puma, Noxa, and BID by heterodimerization (Cheng et al, 2001), and acts on the release of cytochrome *c* by interference with the mitochondrial megapore complex (PT pore) (Shimizu et al, 1999, 2000). Apoptosis by triggering the loss of mitochondrial membrane integrity is the result of intracellular proteolysis mediated by intracellular proteases known as caspases (such as caspase-3, -7, and caspase-9) (Wolf and Green, 1999; Zou et al, 1999; Hengartner, 2000; Kroemer and Reed, 2000). On the other hand, the inhibitor of apoptosis (IAP) family proteins, including cellular IAP-1 (cIAP-1), cIAP-2, X-linked IAP (XIAP), and survivin, were characterized by the presence of the baculoviral IAP repeat, zinc ring finger, and caspase recruitment domain (Deveraux et al, 1997, 1998). These proteins have been shown to inhibit active caspase-3 and -7 directly and to inhibit activation of procaspase-9 (Deveraux and Reed, 1999).

Regarding apoptosis induced by specific COX-2 inhibitors such as celcoxib or NS398 on malignancies including leukemia, some apoptosis signaling pathways have been reported (Nakanishi et al, 2001; Waskewich et al, 2002; Zetterberg et al, 2003). However, the mechanisms of etodolac, COX-2 inhibitor, have not been analyzed in detail yet. In this report, we showed that etodolac were effective against leukemia cells, and it acted in an independent manner as well as other cancers (Sheng et al, 1997; Souza et al, 2000). We chose two COX-2 inhibitors, etodolac and meloxicam, clinically used in Japan. Generally, it has been reported that many COX-2 inhibitors having structures that exploit binding within the COX-2 side-pocket (via sulphonyl, sulphone, or sulphonamide groups) to achieve selectivity, results in inhibition of COX-2 effects (Hawkey, 1999). However, the mechanism of etodolac, which has no sulphonyl, sulphone, or sulphonamide groups, remains unclear. To gain insights into the molecular details of etodolac-induced apoptosis, the expression of anti-apoptotic proteins, the activation of caspases, and the influence of caspase inhibitors were investigated. In addition, relations between bcl-2 and the mitochondrial membrane potentials were investigated after treatment with etodolac in leukemia cells, K562, NB4, U937, HL60, and CEM cells. We investigated the effects of etodolac on the growth of K562 leukemia cells *in vivo*. Moreover, we compared the anti-proliferation effects of etodolac with the stereoisomers of etodolac, R-etodolac and S-etodolac, in leukemia cells. Our data show that apoptosis induced by etodolac is mediated through down-regulation of anti-apoptotic bcl-2 and caspase-9 dependent mitochondrial pathway, and growth inhibition by etodolac is observed *in vivo*. Furthermore, etodolac induced apoptosis more effectively than both R- and S-etodolac. These findings do support additional investigation for the use of etodolac as a therapeutic agent against leukemia.

## II. Materials and methods

### A. Reagents and chemicals

The highly selective COX-2 inhibitors, etodolac, R-etodolac, and S-etodolac, were kindly provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). The highly selective COX-2 inhibitor, meloxicam, was kindly provided by Boehringer Ingelheim (Germany). These drugs were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St Louis, MO), and diluted in culture medium immediately before use. The final concentration of DMSO in all experiments was less than 0.01 %, and all treatment conditions were compared with vehicle controls. 3, 3'-dihexyloxycarbocyanine iodide (DiOC6) was purchased from Molecular Probes (Eugene, OR).

### B. Cell lines and cell culture

NB4 cells were donated by Dr M. Lanotte (Hospital Saint-Louis, Paris, France). HL-60, K562, U937, and CEM cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD). The cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 µg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL, Gaithersburg, MD). All cells were maintained in a humidified 5 % CO<sub>2</sub> atmosphere at 37 °C.

### C. RT-PCR

K562, NB4, U937, HL60, and CEM cells were cultured in 2 ml complete medium containing 1 x 10<sup>6</sup> cells in the presence of etodolac, or meloxicam at 100 µM and incubated at 37 °C. Total RNAs were extracted at 0, 12 h and 16 h after incubation using an RNeasy system (Quiagen, Tokyo, Japan), and 2 µg of total RNAs were reverse transcribed using a 1st strand cDNA synthesis kit (Roche, Indianapolis, IN). PCR was performed using a DNA thermal cycler (model PTC 200; MJ Research, Watertown, MA). Oligonucleotide sequences for each primer are as follows: COX-1, sense 5'-CTTGACCGCTACCACTGTGA-3', antisense 5'-AGAGGGCAGAATACGAGTGT-3'; COX-2, sense 5'-AAGCCTTCTCTAACCTCTCC-3', antisense 5'-TAAGCACATCGCAT-ACTCTG-3'; bcl-2, sense 5'-CGACGACTTCTCCCGCCGGCTACCCG-3', antisense 5'-CCGCATGCTGGGGCCGTACAGTTCC-3'; bcl-xL, sense 5'-TTGGACAATGGACTGGTTG-3', antisense 5'-GTAGAGTGGATGGTCACTG-3'; bax, sense 5'-ATGGACGGTCCGGGGAGCAGCCC-3', antisense 5'-GGTGAGCACTCCCGCCACAAAGAT-3'; bak, sense 5'-TGAAAATGGCTTCGGGGCAAGGC -3', antisense 5'-TCATGATTTGAAGAATCTTCGTACC -3'; and G3PDH; sense 5'-GAACGGGAAGCTCACTGGCATGGC-3', antisense 5'-TGAGGTCCACCACCCTGTTGCTG-3'. PCR conditions of COX-1, COX-2, bcl-xL, and G3PDH were 28 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. PCR conditions of bcl-2, bak and bax were 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1.5 min, and extension at 72 °C for 1.5 min. PCR products were electrophoresed in a 1.5 % agarose gel containing 500 µg/l ethidium bromide and visualized with UV light. In each experiment, RT-PCR was performed in duplicate.

### D. Assay of PGE<sub>2</sub> production

K562, NB4, U937, HL60, and CEM cells (2 x 10<sup>4</sup> per well) were preincubated with 50 or 100 µM etodolac or meloxicam in 24-well plates containing RPMI 1640 medium with 1 % (v/v) FCS at 37 °C in an atmosphere of 5 % CO<sub>2</sub>. After 2 h, the PGE<sub>2</sub>

level in the culture medium was measured using an ELISA kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions.

### E. MTT cell proliferation assay

For the MTT assay, the cells were seeded in 96-well flat-bottomed microplates at a density of  $5 \times 10^4$  per well. Cells were incubated with or without etodolac, or meloxicam at  $37^\circ\text{C}$  for 72 h, and then  $10 \mu\text{l}$  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (Sigma Chemical Co., St. Louis, MO) was added to each well at a final concentration of 1 mg/ml/well. Cells grown in the presence of medium alone were used as controls. After incubation at  $37^\circ\text{C}$  for 4 h, absorbance was measured at a wavelength of 560 nm using a microplate reader.

### F. Apoptosis analysis

DNA content analysis was performed using propidium iodide (PI) staining. Cells were cultured in 2 ml complete medium containing  $1 \times 10^6$  cells in the presence of etodolac, or meloxicam at the indicated concentrations and incubated at  $37^\circ\text{C}$ . After 48 h of incubation, the cells were washed twice with cold PBS, fixed with 70 % ethanol overnight before treatment with  $100 \mu\text{g/ml}$  RNase A, and then stained with  $50 \mu\text{g/ml}$  PI. The relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France).

Cells were cultured in 2 ml complete medium containing  $1 \times 10^6$  cells in the presence of etodolac ( $100 \mu\text{M}$ ), R-etodolac ( $100 \mu\text{M}$ ), S-etodolac ( $100 \mu\text{M}$ ), or R-etodolac ( $100 \mu\text{M}$ ) and S-etodolac ( $100 \mu\text{M}$ ), and incubated at  $37^\circ\text{C}$ . After 48 or 72 h of incubation, the cells were washed twice with cold PBS, fixed with 70 % ethanol overnight before treatment with  $100 \mu\text{g/ml}$  RNase A, and then stained with  $50 \mu\text{g/ml}$  PI. The relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer.

### G. Caspase 3 activation assay

The cells ( $3 \times 10^4$  cells/well) were treated with etodolac or meloxicam at the indicated concentrations during incubation in 96-well plates containing complete medium at  $37^\circ\text{C}$ . After 18 h, the level of caspase activity in the cells was measured using a CaspACE Assay System (Promega, Madison, WI) according to the manufacturer's instructions using a microplate reader.

### H. Western blot analysis

Western analyses of bcl-2, bcl-xL, caspase-9, caspase-8, caspase-7, caspase-3, cIAP-1, and survivin were performed using specific monoclonal antibodies. The leukemia cells were incubated with 50 or  $100 \mu\text{M}$  etodolac or meloxicam for 18 and 24 h, then harvested, washed with cold PBS, and resuspended in lysis buffer containing 0.5 % Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate and 1 mM dithiothreitol supplemented with one Complete Mini protease inhibitor tablet (Boehringer Mannheim, Indianapolis, IN) per 20 ml lysis buffer immediately before use. Samples containing  $50 \mu\text{g}$  protein were added to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer with 5 %  $\beta$ -mercaptoethanol, heated to  $100^\circ\text{C}$  for 2 minutes, and loaded onto 10 % polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 0.5 % milk in PBS for 1 h at room temperature. After being

washed in Tris-buffered saline Tween (TBS-T), the membranes were incubated for 1 h at room temperature with an appropriate dilution of mouse monoclonal anti-bcl-2 antibody (PharMingen, San Diego, CA), rabbit polyclonal anti-bcl-xL antibody (PharMingen), mouse monoclonal anti-caspase-9 antibody (PharMingen), mouse monoclonal anti-caspase-8 antibody (PharMingen), mouse monoclonal anti-caspase-7 antibody (PharMingen), mouse monoclonal anti-caspase-3 (CPP32) antibody (PharMingen), mouse monoclonal anti-cIAP-1 antibody (PharMingen), or rabbit polyclonal anti-survivin antibody (Alpha Diagnostic, San Antonio, TX). After being washed in TBS-T, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Amersham, Arlington Heights, IL) for 1 h and exposed to X-ray film at room temperature. The signal was detected by chemiluminescence using an ECL detection kit (Amersham).

### I. Detection of changes in the mitochondrial membrane potential ( $\Delta\psi$ )

To detect  $\Delta\psi$ , the cells ( $1 \times 10^4$  cells/well) were incubated with 50 and  $100 \mu\text{M}$  etodolac or meloxicam for 16 and 18 h in 24-well plates containing complete medium at  $37^\circ\text{C}$ . After 16 h and 18 h, the cells were labeled with DiOC6 (40 nM in culture medium) at  $37^\circ\text{C}$  for 20 min. After washing in PBS, cellular uptake of DiOC6 was analyzed by flow cytometry.

### J. Flow cytometric evaluation of bcl-2 protein expression

The cells ( $5 \times 10^4$  cells/well) were treated with etodolac or meloxicam at the indicated concentrations during incubation in 24-well plates containing complete medium at  $37^\circ\text{C}$ . After 16 h and 18 h, the cells were fixed and permeabilized by the Fix and Perm Kit (AN DER GRUB, Kaumberg, Austria) according to the manufacturer's instructions. For detection of bcl-2 expression, a FITC-conjugated monoclonal mouse anti-human bcl-2 antibody (DAKO, Glostrup, DK) was used. After washing in PBS, the cells were resuspended in 1.0 ml PBS containing 0.5 % formaldehyde and analyzed by flow cytometry.

### K. *In vivo* tumor growth model

Nude female congenic athymic mice (Charles River, Wilmington MA) were used in human tumor model. They were 4-6 weeks old and weighed 18-20 g at the start of the experiments. Mice received proper care and maintenance in accordance with institutional guidelines. They were injected subcutaneously (s.c.) with  $3 \times 10^7$  K562 cells. Tumors were allowed to grow and establish until they had reached a diameter of 6-8 mm (designated day 0). Animals were then randomized and etodolac (8.0 mg/kg per mouse), etodolac (16.0 mg/kg per mouse), meloxicam (16.0 mg/kg per mouse), or PBS was administered intravenously (i.v.) at day 4, 8, 12, 16, 20, 24. Each group contained three mice aged 5-6 weeks. Tumor growth was monitored by measuring with calipers every 4 days and tumor volume was calculated according to the formula:

$$\text{volume} = L * W^2 / 2,$$

where L is the length (mm) and W is the width (mm).

### III. Results

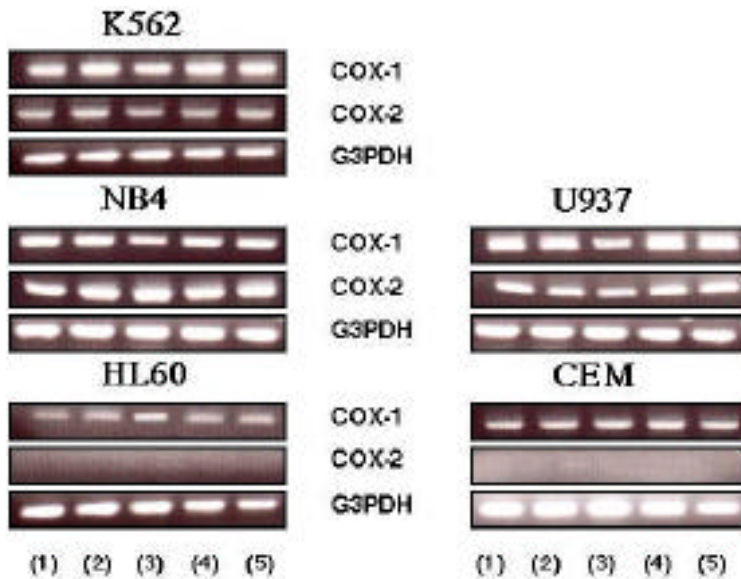
#### A. RT-PCR analysis of COX-1 and COX-2 expression in leukemia cells

As shown in **Figure 1**, the mRNA expression of COX-1 was not significantly different among K562, NB4, U937, HL60, and CEM cells because COX-1 is constitutively expressed in various cells. In contrast, the mRNA expression of COX-2 was detected in K562, NB4, and U937 cells but not in HL60 and CEM cells. K562, NB4, and U937 cells showed similar COX-2 mRNA expression levels. We next examined whether treatment with COX-2 inhibitors, etodolac or meloxicam, influenced COX-2 mRNA expression. Both COX-2 inhibitors did not

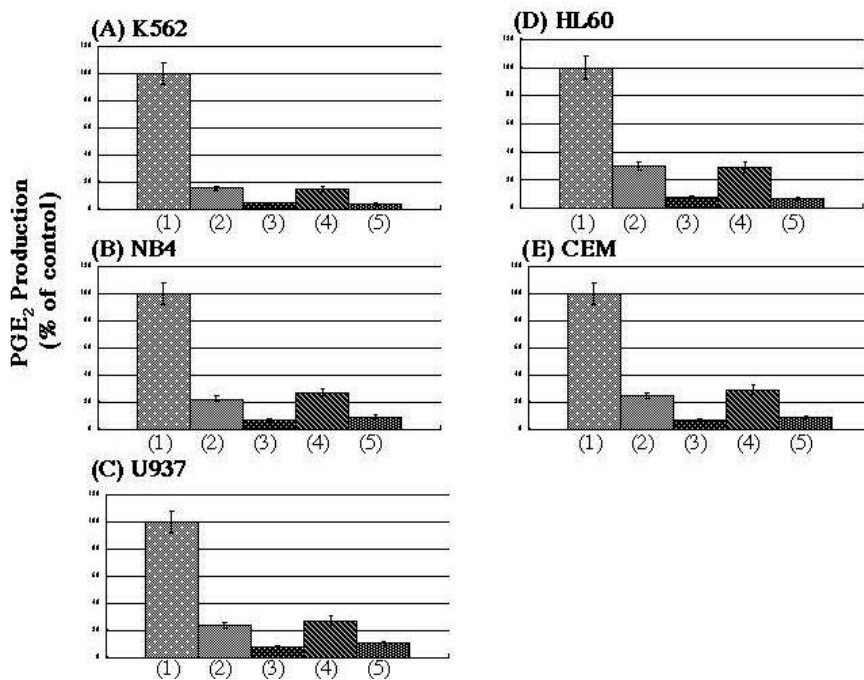
affect COX-2 mRNA expression in K562, NB4, and U937 cells.

#### B. Effects of etodolac and meloxicam on PGE<sub>2</sub> production in leukemia cells

To examine the effects of COX-2 inhibitors on PGE<sub>2</sub> production in K562, NB4, U937, HL60, and CEM cells, cells were treated with etodolac or meloxicam for 2 h. As shown in **Figure 2**, both of COX-2 inhibitors suppressed PGE<sub>2</sub> production in a dose-dependent manner in all leukemia cell lines. No significant differences on inhibition of PGE<sub>2</sub> production by COX-2 inhibitors were observed.



**Figure 1.** RT-PCR analysis of COX-1 and COX-2 mRNA expression in K562, NB4, U937, HL60, and CEM cells. K562, NB4, U937, HL60, and CEM cells were treated with or without etodolac (50 or 100  $\mu$ M) and meloxicam (50 or 100  $\mu$ M) for 16 h. The PCR products were 311 bp in size for COX-1 (upper panel), 533 bp for COX-2 (middle panel), and 320 bp for G3PDH (bottom panel). (1) untreated, (2) treated with 50  $\mu$ M etodolac, (3) treated with 100  $\mu$ M etodolac, (4) treated with 50  $\mu$ M meloxicam, and (5) treated with 100  $\mu$ M meloxicam.



**Figure 2.** Effects of etodolac or meloxicam on the production of PGE<sub>2</sub> in leukemia cells. Cells were treated with etodolac or meloxicam for 2 h and then the PGE<sub>2</sub> level in the culture medium was measured by enzyme immunoassay. The PGE<sub>2</sub> levels in the control cells (untreated K562, NB4, U937, HL60, and CEM cells) were  $3.4 \pm 0.07$ ,  $2.91 \pm 0.06$ ,  $3.24 \pm 0.04$ ,  $2.96 \pm 0.05$  and  $3.59 \pm 0.07$  ng/ml, respectively. Data shown as mean  $\pm$  S.D. in triplicate culture and are representative of three independent experiments. (1) untreated, (2) treated with etodolac (50  $\mu$ M), (3) treated with etodolac (100  $\mu$ M), (4) treated with meloxicam (50  $\mu$ M), (5) treated with meloxicam (100  $\mu$ M).

### C. Effects of COX-2 inhibitors on proliferation of leukemia cells

We examined the effects of COX-2 inhibitors, etodolac and meloxicam, on the proliferation of leukemia cells by MTT assay (Figure 3). K562, NB4, U937, HL60, and CEM cells were incubated with etodolac or meloxicam at the indicated concentrations for 72 h. Etodolac strongly suppressed cell proliferation in a dose-dependent manner. In K562 cells, the growth inhibitory effect of etodolac was observed at 50  $\mu\text{M}$ , and became obvious at 100  $\mu\text{M}$  (Figure 3A). Similar growth inhibition by etodolac was shown in NB4, U937, HL60, and CEM cells, respectively (Figure 3B, C, D, and E). The growth of these cells was completely suppressed at 100  $\mu\text{M}$  etodolac. At concentrations of 100  $\mu\text{M}$  and higher, changes in cell proliferation were not seen (data not shown). In all leukemia cells, growth inhibition by 100  $\mu\text{M}$  etodolac was seen on day 2, and became obvious on

day 2 to 3. In contrast, the growth inhibitory effects of meloxicam were moderate on leukemia cells, K562, NB4, U937 HL60, and CEM cells.

### D. Etodolac induced apoptosis in leukemia cells

All leukemia cells were treated for 24, 48, or 72 h and subsequently stained with propidium iodide and analyzed using flow cytometry (Table 1, Figure 4).

In contrast, after treatment of leukemia cells with meloxicam, induction of apoptosis was slightly observed. Treatment of all leukemia cells with etodolac led to a time- and dose-dependent induction of apoptosis. Doses of 50-100  $\mu\text{M}$  were sufficient to induce apoptotic changes. Moreover, addition of  $\text{PGE}_2$  did not completely prevent etodolac-induced apoptosis (data not shown).

Table 1.

Cell line	% of apoptotic cells (24h)		% of apoptotic cells (48h)		% of apoptotic cells (72h)	
	50 $\mu\text{M}$ etodolac	100 $\mu\text{M}$ etodolac	50 $\mu\text{M}$ etodolac	100 $\mu\text{M}$ etodolac	50 $\mu\text{M}$ etodolac	100 $\mu\text{M}$ etodolac
K562	15.6 $\pm$ 2.6	32.5 $\pm$ 1.8	66.4 $\pm$ 3.1	79.2 $\pm$ 3.9	79.2 $\pm$ 3.6	88.4 $\pm$ 4.3
NB4	19.6 $\pm$ 2.1	37.5 $\pm$ 2.4	64.8 $\pm$ 1.5	84.3 $\pm$ 4.1	80.3 $\pm$ 2.9	94.2 $\pm$ 4.1
U937	20.5 $\pm$ 3.5	38.2 $\pm$ 3.1	76.5 $\pm$ 2.6	79.3 $\pm$ 2.5	80.2 $\pm$ 3.5	89.6 $\pm$ 3.6
HL60	18.3 $\pm$ 1.9	21.5 $\pm$ 1.7	65.6 $\pm$ 2.3	80.4 $\pm$ 3.2	74.6 $\pm$ 3.4	89.3 $\pm$ 4.6
CEM	21.7 $\pm$ 2.3	26.3 $\pm$ 2.2	64.3 $\pm$ 1.9	79.8 $\pm$ 2.9	76.8 $\pm$ 2.7	92.4 $\pm$ 4.1

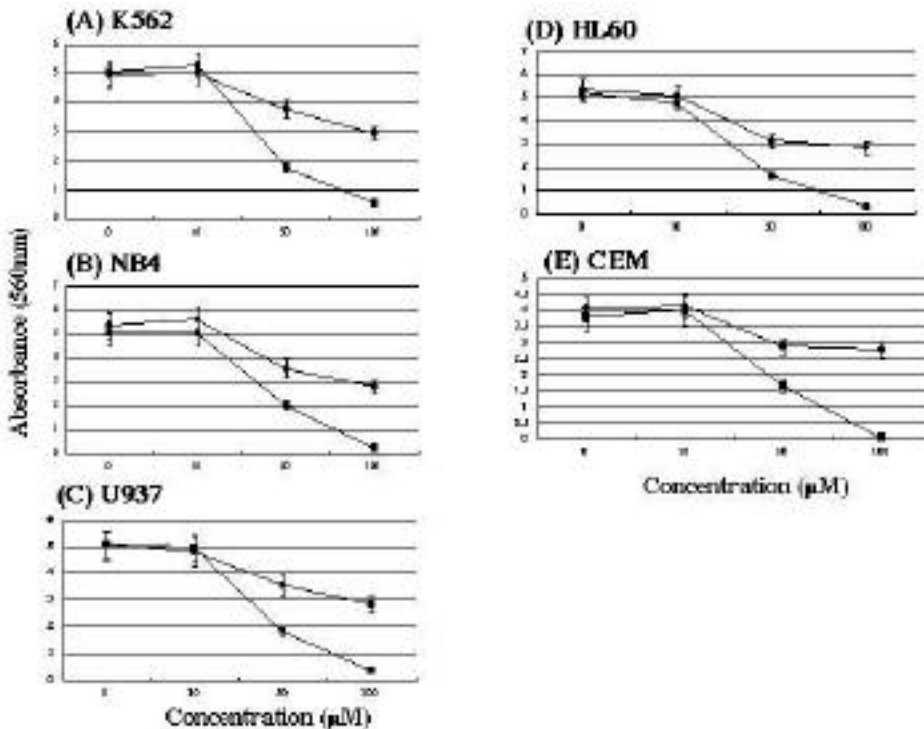
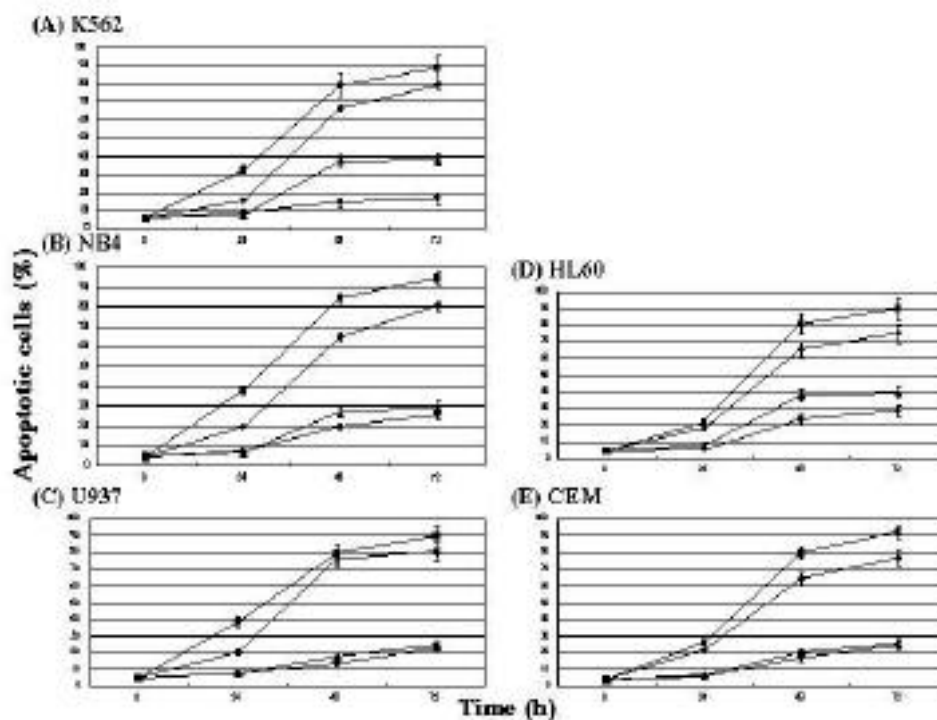


Figure 3. Cell proliferation of K562, NB4, HL60, U937, and CEM cells treated with etodolac or meloxicam. The cells were treated with etodolac or meloxicam at the indicated concentration for 72 h. Cell proliferation was measured by MTT assay. Data represent the mean ( $\pm$  SD) of three independent experiments. Panel (A), (B), (C), (D), and (E) show the inhibition of proliferation in K562, NB4, U937, HL60, and CEM cells, respectively. ■; etodolac, ●; meloxicam.



**Figure 4.** Effects of etodolac or meloxicam on apoptosis of K562, NB4, HL60, U937, and CEM cells. These cells were treated with 50  $\mu$ M, 100  $\mu$ M etodolac, or 50  $\mu$ M, 100  $\mu$ M meloxicam for 24, 48, or 72 h. After treatment, cell were stained with propidium iodide and analyzed by flow cytometry. Data represent the mean ( $\pm$  SD) of three independent experiments. Panel (A), (B), (C), (D), and (E) show the apoptotic cells (%) in K562, NB4, U937, HL60, and CEM cells, respectively. (◆; treated with 50  $\mu$ M etodolac, ■; treated with 100  $\mu$ M etodolac, ●; treated with 50  $\mu$ M meloxicam, ▲; treated with 100  $\mu$ M meloxicam)

### E. Effects of etodolac and meloxicam on caspase-3 activity in leukemia cells

Caspases are responsible for many of the biological and morphological changes that occur during apoptosis. Since caspase-3 is an important effector in apoptosis, we next investigated whether the induction of apoptosis of leukemia cells by etodolac or meloxicam was mediated by the activation of caspase-3. As shown in **Figure 5**, induction of caspase-3 activation was observed at 100  $\mu$ M etodolac, and caspase-3 activity was blocked by incubation with the caspase inhibitor, Z-VAD-FMK (50  $\mu$ M). A 4.3-6.5 fold increase in caspase 3 activity was detected in K562, NB4, U937, HL60, and CEM cells after treatment with 100  $\mu$ M etodolac, and a 2.5-3.9 fold decrease in caspase-3 activity was detected in all leukemia cells by addition of Z-VAD-FMK. In contrast, after 16 h treatment of leukemia cells with meloxicam, the moderate caspase-3 activation (a 2.9-3.9 fold increase) detected as compared with etodolac. Therefore, caspase-3 activity was more strongly detected in treatment with etodolac than meloxicam. Moreover, addition of PGE<sub>2</sub> did not completely prevent etodolac-induced caspase-3 activation (data not shown).

### F. Etodolac decreased the expression of various apoptotic regulatory proteins, bcl-2, bcl-xL, caspase -9, -8, -7, -3, cIAP-1, and survivin

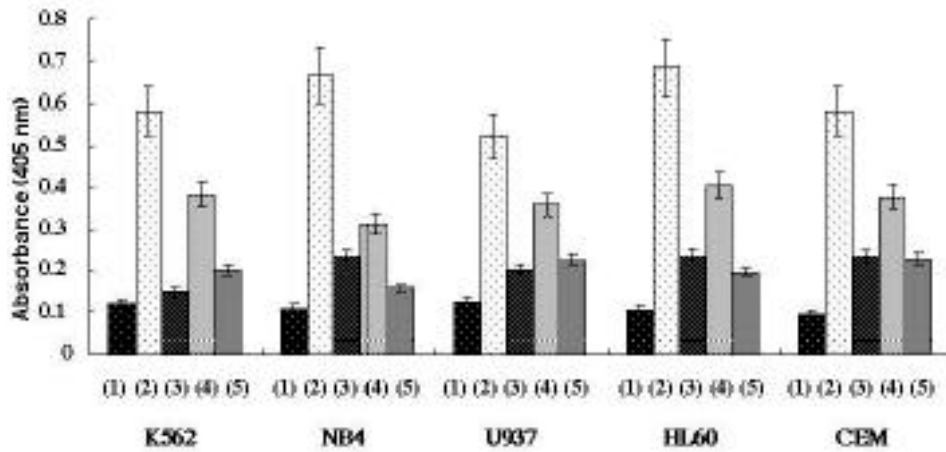
The effects of 24 h treatment with etodolac or meloxicam in leukemia cells were examined in relation to expression of various apoptotic regulatory proteins (**Figure 6**). As shown in **Figure 6A**, treatment of leukemia cells with meloxicam exerted little effect on bcl-2 and bcl-xL protein expression. On the other hand, etodolac treatment resulted in reduction of bcl-2 protein expression in a dose-dependent manner, and exerted little effect on bcl-xL protein expression except for HL60 and U937 cells. Next, we examined the activities of caspase-9, -8, -7, and -3 on effects of etodolac and meloxicam (**Figure 6B**). Procaspace-8 levels remained unchanged with etodolac or meloxicam treatment. Treatment of etodolac resulted in significant cleavage of procaspase-9, -7, and -3 in a dose-dependent manner. In contrast, meloxicam treatment had no effect or slightly reduction on the cleavage of procaspase-9, -7, and -3 in HL60, U937 and CEM cells, or K562 and NB4 cells, respectively. Lastly, little change in expression of c-IAP-1 and survivin was noted with meloxicam treatment. On the other hand, etodolac treatment resulted in reduction of c-IAP-1 and survivin protein expression in a dose-dependent manner (**Figure 6C**). Thus, treatment leukemia cells with etodolac induced

down-regulation of the anti-apoptotic proteins, and was associated with activation of caspase cascades.

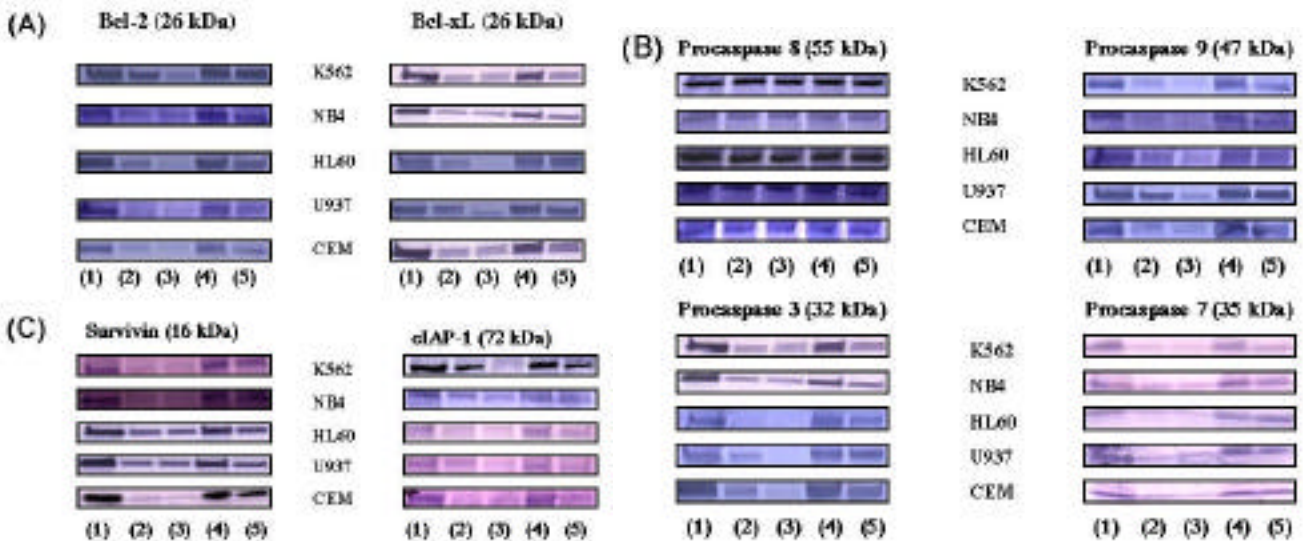
### G. Changes of the mitochondrial membrane potential ( $\psi_m$ ) in leukemia cells by treatment with etodolac or meloxicam

In preceding the activation of caspases, the disruption of the mitochondrial membrane potential was investigated in COX-2-induced apoptosis. The breakdown of the

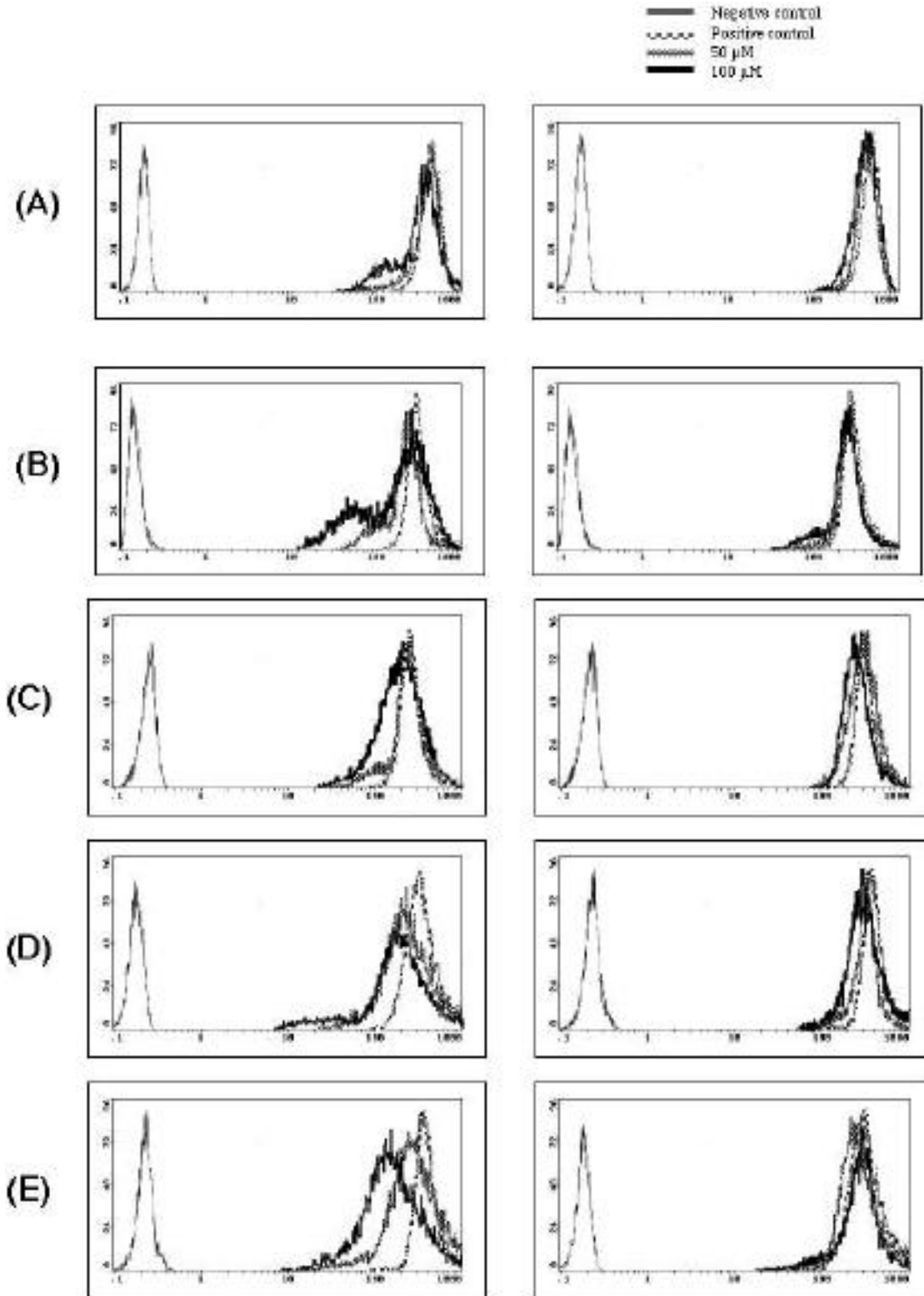
mitochondrial membrane potential was determined by DiOC6 uptake and subsequent flow cytometry. After 16 h of treatment of all leukemia cells with etodolac or meloxicam, no substantial changes of the mitochondrial membrane potential were shown (data not shown). However, after 18 h of treatment with etodolac, the DiOC<sub>6</sub> fluorescences were significantly reduced in a dose-dependent manner in these cells (Figure 7).



**Figure 5.** Effects of etodolac or meloxicam on caspase 3 activation in K562, NB4, U937, HL60, and CEM cells. For caspase 3 activation, and investigating whether etodolac - or meloxicam - induced activation of caspase 3 was reversed by addition of a caspase inhibitor, Z-VAD-FMK, these cells were treated with etodolac (100 μM) or meloxicam (100 μM) with or without 50 μM Z-VAD-FMK for 16 h and then collected. Cell lysates were analyzed for caspase - 3 activation. The level of caspase 3 activity in the cells was measured using a CaspACE Assay System by using a microplate reader. Data represent the mean (± SD) of three independent experiments. (1) untreated, (2) etodolac (100 μM), (3) etodolac (100 μM) and Z-VAD-FMK (50 μM), (4) meloxicam (100 μM), (5) meloxicam (100 μM) and Z-VAD-FMK (50 μM).



**Figure 6.** Western blot analysis of effects of etodolac and meloxicam on the expression of various apoptotic regulatory proteins, bcl-2, bcl-xL, caspase -9, -8, -7, -3, cIAP-1, and survivin. K562, NB4, HL60, U937, and CEM cells were treated with etodolac or meloxicam for 24 h, after which cells were lysed, proteins separated by SDS-PAGE, and Western analysis performed to monitor expression of various proteins. (A) bcl-2 (left panels) and bcl-xL (right panels), (B) Procaspase-8 (left upper panels), Procaspase-9 (right upper panels), Procaspase-3 (left bottom panels), and Procaspase-7 (right bottom panels), and (C) survivin (left panels) and cIAP-1 (right panels). (1) Cells cultured without agents, (2) cells cultured with 50 μM etodolac, (3) cells cultured with 100 μM etodolac, (4) cells cultured with 50 μM meloxicam, and (5) cells cultured with 100 μM meloxicam for 24 h.



**Figure 7.** Effects of etodolac and meloxicam on the mitochondrial membrane potential of leukemia cell lines, K562, NB4, U937, HL60, and CEM cells. Cells were treated with 50, or 100  $\mu\text{M}$  etodolac (left lane panels) or 50, or 100  $\mu\text{M}$  meloxicam (right lane panels) for 18 h. To determine the mitochondrial membrane potential, cells were stained with DiOC<sub>6</sub> and analyzed by flow cytometry. (A) K562, (B) NB4, (C) U937, (D) HL60 and (E) CEM cells.

After 24 h of the treatment with etodolac, remarkable reduction of DiOC<sub>6</sub> fluorescence were observed, indicating breakdown of the mitochondrial membrane potential in

these cells (data not shown). These results demonstrated that etodolac treatment induced a time-and dose-dependent breakdown

of the mitochondrial potential. In contrast, no significant breakdown of the mitochondrial membrane potential was observed in these cells treated with meloxicam.

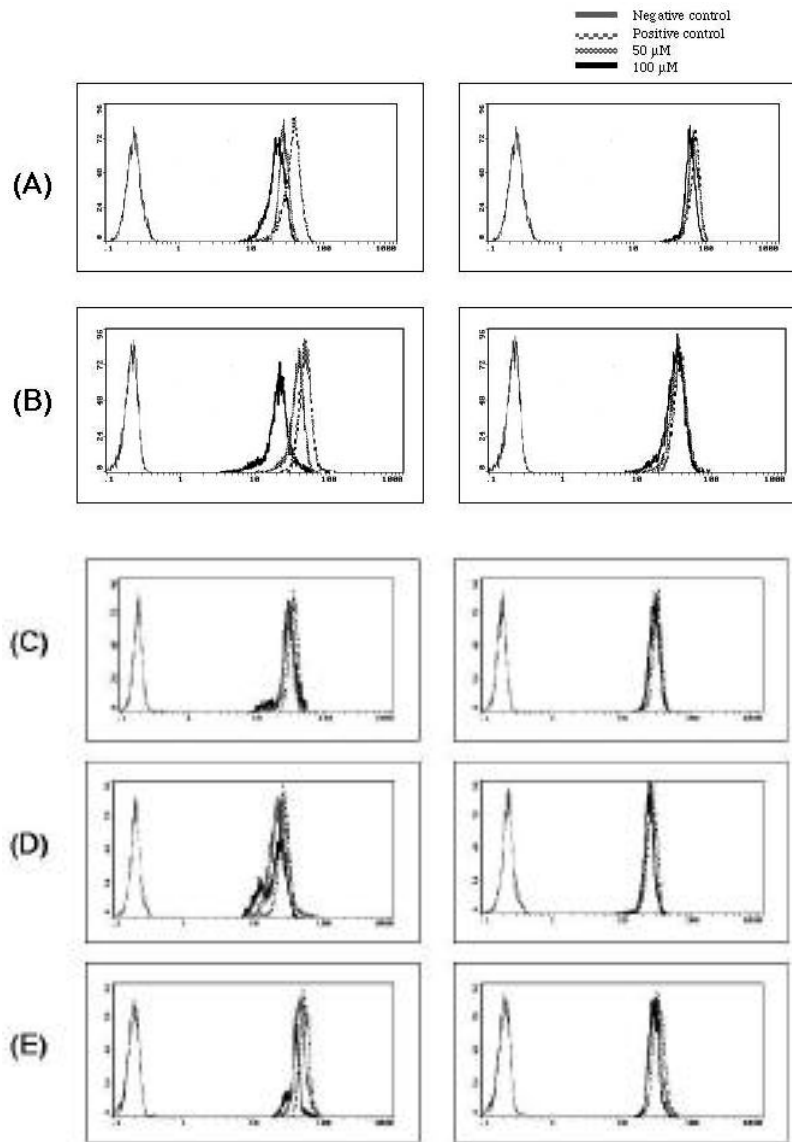
### H. Effects of COX-2 inhibitors on expression of anti-apoptotic bcl-2 protein in leukemia cells

Since the bcl-2 protein is reported to have an important role to maintain the mitochondrial membrane potential, we examined whether treatment with etodolac or meloxicam changed bcl-2 protein expression in leukemia cells by flow cytometry (Figure 8). After 16 h of treatment of leukemia cells with etodolac, bcl-2 down-regulation preceded the breakdown of the mitochondrial membrane potential. In particular, on the treatment of K562 and NB4 cells with 100  $\mu$ M etodolac,

remarkable reduction of bcl-2 expression was observed. Moreover, remarkable reduction of bcl-2 expression in all leukemia cells was observed after 18 and 24 h of the etodolac treatment (data not shown). Etodolac treatment also induced a time-and dose-dependent down regulation of the bcl-2 expression. In contrast, the meloxicam treatment led to no detectable change in the intracellular bcl-2 expression in leukemia cells.

### I. RT-PCR analysis of bcl-2, bcl-xL, bak and bax mRNA in leukemia cells treated with etodolac

Next, we investigated expression of antiapoptotic (bcl-2 and bcl-xL) and proapoptotic (bax and bak) mRNAs in leukemia cells treated with etodolac by RT-PCR (Figure 9).



**Figure 8.** Effects of etodolac and meloxicam on intracellular bcl-2 protein expression of leukemia cell lines, K562, NB4, U937, HL60, and CEM cells. Cells were treated with 50, or 100  $\mu$ M etodolac (left lane panels) or 50, or 100  $\mu$ M meloxicam (right lane panels) for 16 h. After treatment, cells were washed, permeabilized, stained with a FITC-conjugated monoclonal mouse anti-human bcl-2 antibody, and analyzed by flow cytometry. (A) K562, (B) NB4, (C) U937, (D) HL60 and (E) CEM cells.

All cell lines were treated with 50 or 100  $\mu\text{M}$  etodolac for 12 h. All cell lines had relatively equal amplification of the housekeeping gene G3PDH, implying that equal amounts of each mRNA were used in these experiments. Bcl-2 mRNA expression was decreased in all cell lines treated with 50 and 100  $\mu\text{M}$  etodolac, and remarkable reduction of bcl-2 mRNA in all leukemia cells was observed after 100  $\mu\text{M}$  etodolac treatment. Etodolac treatment also induced a dose-dependent reduction of the bcl-2 mRNA expression. In contrast, no significant reduction of bcl-xL mRNA was detected in these cells after 12 h of treatment of etodolac. Interestingly, bax mRNA expression was also decreased in all cell lines as well as bcl-2 mRNA, but no significant reduction of bak mRNA expression was detected in all leukemia cells. Etodolac treatment induced the reduction of bcl-2 and bax mRNA following breakdown of mitochondrial membrane potential in leukemia cells.

#### J. *In vivo* K562 cell growth inhibition by etodolac treatment

We confirmed the use of etodolac in a leukemia cell line *in vivo*. K562 cells were implanted s.c. into nude mice. Etodolac (8.0 mg/kg or 16.0mg/kg), meloxicam (16.0 mg/kg), or PBS were injected i.v. via tail vein at day 4, 8, 12, 16, 20, 24. As shown in **Figure 10**, there were significant differences in K562 cell growth in etodolac treated mice compared with meloxicam or PBS treated mice at day 16 after the initial injection (day 4). Moreover, etodolac (16.0 mg/kg) in K562 cell growth inhibition was a marked antitumor effect compared with 8.0 mg/kg etodolac. It was reported that when 400mg (8.0 mg/kg) etodolac was administered to adult human orally, the serum concentrations achieved were  $\sim 75 \mu\text{M}$  (21  $\mu\text{g/ml}$ ). These data demonstrate that etodolac reduces the growth of K562 leukemia cells *in vivo*.

#### K. Effects of etodolac or stereoisomers of etodolac (R-etodolac or S-etodolac) on apoptosis of K562, NB4, HL60, U937, and CEM cells.

All leukemia cells were treated for 48 or 72 h and subsequently stained with propidium iodide and analyzed using flow cytometry (**Table 2**). In contrast, after

treatment of leukemia cells with 100  $\mu\text{M}$  R- or S-etodolac, induction of apoptosis was slightly observed compared with etodolac. Interestingly, induction of apoptosis with the combination of R-and S-etodolac was not significantly observed. It was demonstrated that etodolac, which consists of R- and S-etodolac, induced apoptosis, whereas simple mixture of R- and S-etodolac significantly did not.

#### IV. Discussion

The aim in this study was to investigate how etodolac induced apoptosis in leukemia cells. The data presented here provide novel insights into the molecular mechanisms of it. There are many COX-2 inhibitors, that have sulphonyl, sulphone, or sulphonamide groups, and in this study, we used etodolac and meloxicam, which have quite similar potency for inhibition of the COX-2 enzyme. Interestingly, etodolac has no sulphonyl, sulphone, or sulphonamide groups, and is different from other COX-2 inhibitors. Recent reports have shown that COX-2 is a key enzyme, and promotes angiogenesis, inflammation, cellular adhesion, growth, differentiation and apoptosis (Eberhart and Dubois, 1995; Tsujii and DuBois, 1995). If COX-2 is a relevant target in leukemia cells, COX-2 inhibitors should be effective in inhibiting the proliferation of leukemia cells. We showed that etodolac strongly induces apoptosis in leukemia cells, K562, NB4, U937, HL60, and CEM cells. However, our data showed that COX-2 mRNA expression was not detected in both HL-60 and CEM cells, while that in K562, NB4, and U937 cells was detected in same level. The effects of etodolac-induced apoptosis were found to be strong and similar for both COX-2 positive and negative leukemia cell lines, and there were no significant differences. In contrast, meloxicam affected moderate induction of apoptosis in leukemia cells. These differences between etodolac and meloxicam were evident in MTT proliferation assays as well as apoptosis assays. It is generally recognized that COX-2 inhibitors exert their actions via blocking PG synthesis by direct COX-2 inhibition (Fujita et al, 2001). Our study showed that etodolac or meloxicam significantly inhibited PGE<sub>2</sub> production. However, addition of PGE<sub>2</sub> did not rescue the etodolac-induced apoptosis (data not shown). Therefore,

**Table 2.**

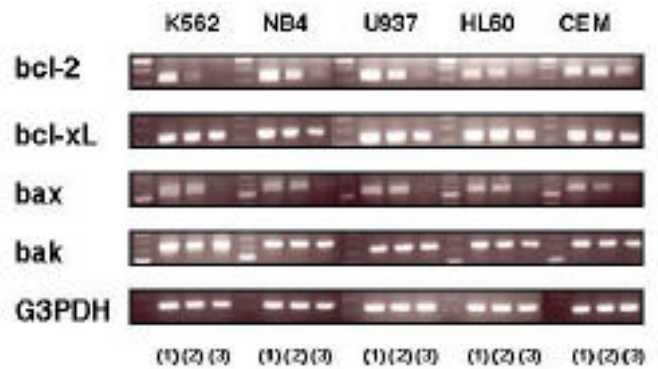
Cell line	% of apoptotic cells (48h)	% of apoptotic cells (72h)
	100 $\mu\text{M}$ etodolac	100 $\mu\text{M}$ etodolac
K562	80.2 $\pm$ 6.2	89.8 $\pm$ 6.2
NB4	85.3 $\pm$ 5.5	95.2 $\pm$ 4.6
U937	81.1 $\pm$ 4.9	91.2 $\pm$ 6.2
HL60	81.2 $\pm$ 6.2	88.3 $\pm$ 5.3
CEM	80.6 $\pm$ 4.9	94.2 $\pm$ 6.7

whether COX-2 inhibitors block proliferation of cancer cells or induce apoptosis solely by inhibiting PG synthesis has not been clarified. Our data showed that etodolac directly down-regulated bcl-2 expression and induced caspase -3-dependent apoptosis in leukemia cells. Our findings suggested that there were COX-2 independent pathways in etodolac-induced apoptosis.

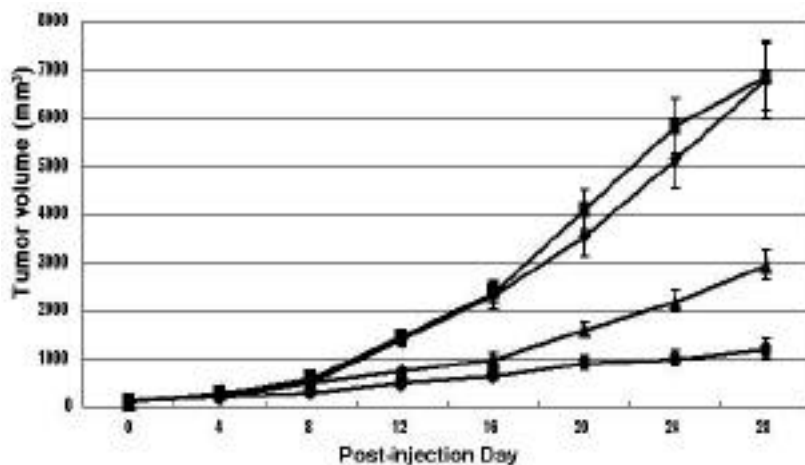
In apoptosis, ionizing radiation, UV light, heat shock, kinase inhibitors, and anti-cancer drugs have all been shown to induce apoptosis through bcl-2-regulated mitochondrial pathway (Strasser et al, 1995; Belka et al, 2000; Ochs and Kaina, 2000; Jendrossek et al, 2002). Bcl-2 has anti-apoptotic functions and decreases of bcl-2 protein expression affect the life-span of cells (Guenal et al, 1997; Li et al, 2001; Huigsloot et al, 2002). Indeed, in this study, etodolac induced some cellular events, including down-regulation of bcl-2 mRNA and protein expression, breakdown of the mitochondrial membrane potential, and caspase-9, -7, and -3 activation, which all are indicative for the involvement of mitochondrial apoptosis pathways. We showed that decreases of bcl-2 triggered by etodolac treatment induced activation of caspase-9, -7 and -3 but not caspase-8. These caspases activation preceded etodolac-induced apoptosis, indicating the mitochondrial-mediated caspase activation (Leoni et al, 1998). Experiments with caspase inhibitor, Z-VAD-FMK, confirmed this event, and Z-VAD-FMK significantly reduced apoptosis. These findings indicate the activation of caspase-9, -7, and -3 is important for etodolac-induced apoptosis, whereas caspase-8 is not essential. In addition, decrease of cIAP-1 and survivin was shown in leukemia cells treated with etodolac. These events might enhance the induction of apoptosis by etodolac.

Etodolac has been reported to consist of stereoisomers, R- and S- etodolac (Brocks et al, 1991). S-etodolac is a specific COX inhibitor, while R-etodolac lacks COX inhibitory activity (Brocks et al, 1992; Mignot et al, 1996). However, both stereoisomers have no

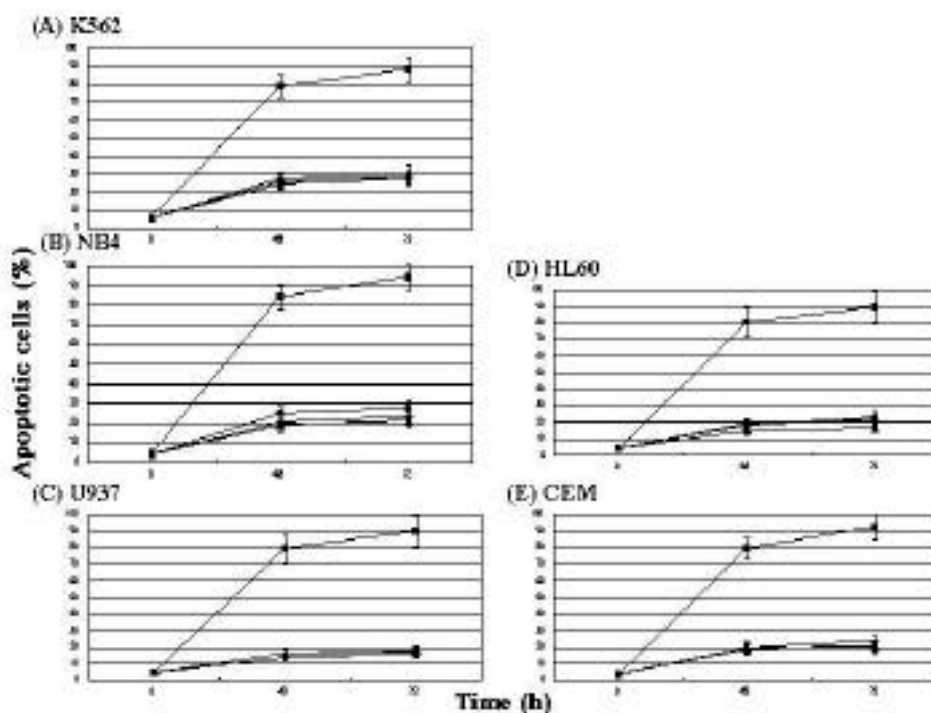
significant differences on effects of apoptosis induction. Therefore, effects of apoptosis induction have been reported to be independent COX inhibition pathway, and R- etodolac has been used in clinical trials in prostate cancer and B-chronic lymphocytic leukemia (CLL) (Adachi et al, 2004). *In vitro* in CLL, multiple myeloma (MM), and lymphoma cells, etodolac has reported to induce apoptosis (Adachi et al, 2000; Leoni et al, 2001; Leoni et al, 2002; Nardella and LeFevre, 2002). R-etodolac (SDX-101; Salmedix Inc) displayed an IC50 ranging from 180 to 300  $\mu$ M in primary CLL cells (Adachi et al, 2004). In CLL, SDX-101 is currently being developed in phase II clinical trials. The activity in lymphoma cell lines tested ranged from 140 (with diffuse large B cell lymphoma, SUDHL-9 cells) to 320  $\mu$ M (for Burkitt's lymphoma, Ramos and Raji cells). MM cell lines displayed an IC50 of about 150  $\mu$ M in RPMI8226 and 350  $\mu$ M in U266 cells (Nardella and LeFevre, 2002).



**Figure 9.** RT-PCR analysis of bcl-2, bcl-xL, bax, and bak mRNA expression levels in K562, NB4, U937, HL60, and CEM cells by treatment of etodolac. K562, NB4, U937, HL60, and CEM cells were untreated and treated with 50 or 100  $\mu$ M etodolac for 12 h. (1) untreated, (2) treated with 50  $\mu$ M etodolac and (3) 100  $\mu$ M etodolac.



**Figure 10.** Etodolac inhibits the *in vivo* growth of K562 cells.  $3 \times 10^7$  K562 cells were injected s.c. into the dorsal flank of nude mice. Etodolac ( $\blacktriangle$ , 8.0 mg/kg;  $\bullet$ , 16.0 mg/kg) and meloxicam ( $\blacklozenge$ , 16.0 mg/kg) were administered on day 4, 8, 12, 16, 20 24. All drugs were administered i.v.  $\blacksquare$ , control animals.



**Figure 11.** Effects of etodolac, R-etodolac, S-etodolac, or the combination of R- and S-etodolac on apoptosis of K562, NB4, HL60, U937, and CEM cells. These cells were treated with 100  $\mu$ M etodolac, 100  $\mu$ M R-etodolac, 100  $\mu$ M S-etodolac, or the combination of 100  $\mu$ M R- and 100  $\mu$ M S-etodolac for 48 or 72 h. After treatment, cell were stained with propidium iodide and analyzed by flow cytometry. Data represent the mean ( $\pm$  SD) of three independent experiments. Panel (A), (B), (C), (D), and (E) show the apoptotic cells (%) in K562, NB4, U937, HL60, and CEM cells, respectively. (■; treated with 100  $\mu$ M etodolac, ●; treated with 100  $\mu$ M R-etodolac, ▲; treated with 100  $\mu$ M S-etodolac, ◆; treated with the combination of 100  $\mu$ M R-etodolac and 100  $\mu$ M S-etodolac)

It has been reported that no correlation between over-expression of bcl-2 and other anti-apoptotic bcl-2 family members. Sensitivity to SDX-101 was observed, and the mechanism of action of SDX-101 studied in primary CLL cells involved the down-regulation of the anti-apoptotic protein Mcl-1, the activation of the PPARs, and the induction of NOR1, an orphan nuclear receptor that has been associated with apoptosis. However, in our study, we found the down-regulation of bcl-2 in leukemia cells, K562, NB4, U937, HL60, and CEM cells, treated with etodolac, which contains both R- and S-etodolac, by flow cytometry and subsequently, collapse of mitochondrial membrane potential. After 16 h treatment with etodolac, in which we could not detect the changes of mitochondrial membrane potential and both bcl-2 and bcl-xL protein by flow cytometry and western blotting analysis, respectively, while we could detect slight differences of cytoplasm bcl-2 protein by flow cytometry. After 12 h treatment with etodolac, bcl-2 and bax mRNA level decreased in a dose dependent manner, while bcl-xL and bak mRNA level unchanged. Therefore, these findings suggest that etodolac induce the down-regulation of bcl-2 in leukemia cells, and etodolac-relating apoptosis is regulated by the reduction of bcl-2 mRNA and the maintenance of bak mRNA. Bak and bax may have a proapoptotic function that is independent on their ability to heterodimerize with bcl-2 and bcl-xL proteins. In etodolac-induced apoptosis, it might be important to remain bak mRNA expression. Future work will focus on the mechanism of etodolac-induced bcl-2 mRNA down regulation. Moreover, we also detected the

reduction of bcl-xL protein, whereas did not detect changes of bcl-xL mRNA. These data might indicate that the effects of etodolac were attributed to the instability or degradation of bcl-xL protein.

The i.v. administration of etodolac at doses of 8.0–16.0 mg/kg resulted in significant and dose-related growth inhibition of K562 leukemia cells compared to PBS or meloxicam treatments, and the toxicity or pronounced morbidity was not observed.

Finally, we investigated the effects of R-etodolac, S-etodolac, and the combination of R- and S-etodolac compared to etodolac in leukemia cells, K562, NB4, U937, HL60, and CEM cells. After treatment with 100  $\mu$ M R- etodolac, S- etodolac, and the combination of R- and S-etodolac, the proliferation of leukemia cells was slightly inhibited, while etodolac significantly inhibited the proliferation of leukemia cells at 100  $\mu$ M. Etodolac was compounded chemically, and 100  $\mu$ M racemate of etodolac contains 50  $\mu$ M R-etodolac and 50  $\mu$ M S-etodolac. The differences between the combination of R- and S-etodolac and racemate of etodolac on the mechanisms of the inhibition of cell proliferation are unknown. When racemate was added, changes in the joint style to receptors might arise, and synergistic effects might be pulled out. When R- and S-etodolac was mixed before addition into a well, the inhibition effects of the combination and rasemate of etodolac were same grade.

In conclusion, our findings indicate etodolac-induced apoptosis follows a bcl-2 dependent

mitochondrial pathway, but COX-2 independent pathway in various leukemia cell lines. Moreover, etodolac more effectively induce apoptosis than R- and/or S-etodolac.

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