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A NOVEL CELECOXIB DERIVATIVE POTENTLY INDUCES APOPTOSIS OF HUMAN SYNOVIAL FIBROBLASTS.

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List of abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; RA, rheumatoid arthritis; PG, prostaglandin; FAP, familial adenomatous polyposis; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; IL-1β, interleukin-1β; FBS, fetal bovine serum; OA, osteoarthritis; BrdU, 5-bromo-2'-deoxyuridine; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; BSA, bovine serum albumin.

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ABSTRACT

We have already demonstrated that celecoxib, a selective cyclooxygenase (COX)-2 inhibitor, has a pro-apoptotic effect on synovial fibroblasts obtained from patients with rheumatoid arthritis (RA). Here we report on the development of two novel derivatives of celecoxib,

N-(2-aminoethyl)-4-[5-(4-tolyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (TT101) and 4-[5-(4-aminophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (TT201), including whether these compounds have a pro-apoptotic effect on synovial fibroblasts. Synovial fibroblasts were harvested from the synovial tissues of patients with RA or osteoarthritis (OA). Cell proliferation and cell viability were assessed by incorporation of 5-bromo-2'-deoxyuridine and by the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt assay, respectively. Apoptosis was detected by identification of DNA fragmentation, and activation of caspase-3 was detected by addition of a caspase-3 substrate to cell lysates. Production of prostaglandin E_2 by RA synovial fibroblasts was analyzed by enzyme-linked immunosorbent assay. TT101 inhibited the proliferation of RA and OA synovial fibroblasts in a concentration-dependent manner. It caused a marked decrease of cell viability and induced DNA fragmentation more potently than either celecoxib or SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-l-yl]benzenesulfonamide). TT101 also increased caspase-3 activity. The order of potency of the COX-2 inhibitory activity of these drugs in RA synovial fibroblasts was celecoxib = SC-236 > rofecoxib > TT201 > TT101. In conclusion, we developed TT101 with about a 5- to 10-fold stronger pro-apoptotic effect on RA and OA synovial fibroblasts compared to that of celecoxib. Although the mechanism of action of TT101 remains unclear, it may have potential as a novel anti-rheumatic agent.

Celecoxib is one of the nonsteroidal anti-inflammatory drugs (NSAIDs) that selectively inhibit cyclooxygenase (COX)-2. It is widely used for treatment of rheumatoid arthritis (RA) with the expectation that an anti-inflammatory effect will result from inhibiting the production of prostanoids, like prostaglandin (PG) E_2 through the suppression of COX-2 activity. On the other hand, we previously demonstrated that celecoxib induces the apoptosis of synovial fibroblasts obtained from patients with RA (Kusunoki et al., 2002).

Apoptosis is considered to be one of the mechanisms regulating autoimmune diseases such as RA (Thompson, 1995). In the pathogenesis of RA, it is thought that the normal balance between proliferation and apoptosis of synovial fibroblasts is lost, leading to hyperplasia of these fibroblasts (Perlman et al., 2001). Activated synovial cells cause growth of synovium in the articular cavity along with angiogenesis, invade the adjacent bone, promote production of inflammatory cytokines by inflammatory cells, and cause cartilage and bone destruction (Ospelt et al., 2004). Therefore, it has been shown that stimulation of the apoptosis of synovial fibroblasts might be useful for the treatment of RA (Nishioka et al., 1998; or Baier et al., 2003).

Recently, celecoxib was reported to cause a significant reduction in the number of colorectal polyps in patients with familial adenomatous polyposis (FAP) (Steinbach et al., 2000) and it has attracted attention as an antiproliferative agent in animal and cell culture studies. We have previously shown that celecoxib induces the apoptosis of colorectal carcinoma cells (Yamazaki et al., 2002). In addition, several studies conducted by other investigators have demonstrated that celecoxib suppresses the proliferation of various cells by inducing apoptosis (Reviewed by Xiong, 2004; Sandler and Dubinett, 2004; Arun and Goss, 2004; Kismet et al., 2004; Sinicrope and Gill, 2004; or Myers et al., 2001), suggesting that the pro-apoptotic action of celecoxib may be useful for the chemoprevention of tumorigenesis (Sinicrope et al., 2004; or Kismet et al., 2004). These effects may represent an action that is

unique to the drug celecoxib, rather than being a class effect of COX-2 inhibitors.

In our *in vitro* study (Kusunoki et al., 2002; and Yamazaki et al., 2002), the concentration of celecoxib required to induce apoptosis of synovial fibroblasts obtained from patients with RA and colon adenocarcinoma cells was slightly higher than the blood level of the drug achieved in healthy individuals (McAdam et al., 1999). Therefore, for adequately pro-apoptotic activity to achieve an anti-rheumatic effect in clinical use, celecoxib may need to be used administered at higher dose levels than those employed clinically. Accordingly, we have attempted to develop a drug with more potent apoptosis-inducing activity for this purpose.

METHODS

Materials

We synthesized N-(2-aminoethyl)-4-[5-(4-tolyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (TT101) and 4-[5-(4-aminophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (TT201). The purity of TT101 was > 99%, as assessed by high-performance liquid chromatography (HPLC) under the following conditions; MeOH solution (1 mg/mL) was injected into the L-column ODS (4.6 x 150 mm), and the mobile phase was MeCN/20 mM phosphate buffer (pH 6.5) (35:65) at a flow rate of 1 mL/min. TT101 was detected with a UV detector at a wavelength of 254 nm and a retention time of 21.2 minutes. ^{1}H NMR (dimethyl sulfoxide (DMSO)-d_6), δ 2.31 (s, 3H), 2.50 (br, 5H), 2.76 (t, J=7.0 Hz, 2H), 7.20 (br, 5H), 7.55 (dd, J=2.0, 6.0 Hz, 2H), 7.84 (dd, J=2.0, 6.0 Hz, 2H). The purity of TT201 was > 99%, as assessed by HPLC under the following conditions; MeOH solution (1 mg/mL) was injected into the L-column ODS (4.6 x 150 mm), and the mobile phase was MeCN/20 mM phosphate buffer (pH 6.5) (1:1) at a flow rate of 1 mL/min. TT201, as previously reported by Penning et al. (Penning et al., 1997), was detected with a UV detector at a wavelength of 254 nm and a retention time of 8.6 minutes (mp, 202-203 °C). Celecoxib and 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-l-yl]benzene sulfonamide (SC-236) were kindly supplied by Pfizer Japan Inc. (Tokyo, Japan). Rofecoxib was synthesized as reported elsewhere (International Patent Publication WO95/00501 or Kato et al., 2001).

Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), and Z-LEHD-FMK (a caspase-9 inhibitor) were purchased from R&D Systems, Inc. (Minneapolis, MN). Interleukin (IL)-1β was purchased from Techne Corporation (Minneapolis, MN). The test drugs were dissolved in DMSO (the final concentration of DMSO in all cultures was 0.1 % (v/v)). RPMI-1640 medium, penicillin-streptomycin solution, fetal bovine serum (FBS),

and trypsin-EDTA were obtained from Gibco BRL (Gaithersburg, MD). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Culture of human synovial fibroblasts

Synovial fibroblasts were prepared from harvested synovial tissues, as described previously (Kawai et al., 1998). The synovial tissues were obtained during the performance of total knee or hip replacement in patients with RA or osteoarthritis (OA) who fulfilled the respective criteria of the American College of Rheumatology (Arnett et al., 1988; Altman et al., 1986; and Altman et al., 1991). The protocol for the study was approved by the Ethics Committees of St. Marianna University and Toho University. All patients gave written informed consent for their tissues to be used in for this research. The synovial tissues were digested for 2 hours in 0.2 % (w/v) bacterial collagenase (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), and the synovial cells thus obtained were suspended in RPMI-1640 medium with 10% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Incubation was performed at 37 °C in 5 % CO_2 for several days, after which nonadherent cells were removed. The fibroblast-like adherent cells were cultured in RPMI-1640 medium containing 1 % (v/v) FBS under a 5 % CO_2 atmosphere and were used as synovial fibroblasts within 2 passages. These adherent cells contained no T cells ($CD3^+$) or macrophages/monocytes ($CD14^+$) on two-color immunofluorescence and flow cytometry (Kusunoki et al., 2002).

Cell proliferation

The proliferative activity of cultured human synovial fibroblasts was estimated from the uptake of 5-bromo-2'-deoxyuridine (BrdU). Cells (1×10^4 /well) were exposed to the test drugs during culture in 96-well plates under the conditions described above. After 24 hours, BrdU (10 μM) was added to the medium, and the cells were incubated for another 18 hours. Then the cells were fixed and nuclear incorporation of BrdU was measured by using a cell proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany) in accordance with the

manufacturer's instructions. Each measurement was performed in triplicate, and the results are presented as percentages relative to the value for untreated control cultures.

Cell viability

Human synovial fibroblasts in 96-well culture plates (2×10^4 cells/well) were exposed to the test drugs under the conditions described above. After 24 hours, cell viability was determined by measuring mitochondrial NADH-dependent dehydrogenase activity with a Cell Counting kit (Dojindo Laboratories, Kumamoto, Japan) that employed a sulfonated tetrazolium salt and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzenedisulfonate (WST-1), in accordance with the manufacturer's instructions. Each measurement was performed in triplicate, and the results are presented as percentages relative to the value for untreated control cultures.

DNA fragmentation

Human synovial fibroblasts in 96-well culture plates (2×10^4 cells/well) were exposed to the test drugs under the conditions described above. After 24 hours, cytoplasmic DNA fragmentation (an indicator of apoptosis) was detected by using a DNA cell death detection ELISA^{PLUS} kit (Roche Diagnostics) in accordance with the manufacturer's instructions. Each measurement was performed in triplicate, and the results are presented relative to that for untreated control cultures. Cell morphology was also examined under a light microscope (BX51, Olympus Optical Co., Ltd., Nagano, Japan) at a magnification of $100\times$.

Caspase activity

RA synovial fibroblasts were incubated in tissue culture flasks in the presence or absence of TT101 and/or Z-IETD-FMK and Z-LEHD-FMK under the conditions described above. After 24 hours, cellular caspase-3 activity was measured by using the CaspACE™

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assay system (Promega Corporation, Madison, WI) in accordance with the manufacturer's instructions. Then the cells were washed in ice-cold phosphate-buffered saline (PBS, 9.57 mM, pH 7.35-7.65, Takara Shuzo Co., Ltd., Shiga, Japan) and resuspended in cell lysis buffer. After lysis of the cells by freezing and thawing, the lysates were centrifuged and the supernatants were used as cell extracts. The protein content of each extract was determined by the bicinchoninic acid (BCA) protein assay method (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as the standard, and the protein content of was adjusted to 1 mg/mL.

Effect of caspase inhibitors on DNA fragmentation induced by TT101

RA synovial fibroblasts in 96-well culture plates (2×10^4 cells/well) were exposed to Z-DEVD-FMK, Z-IETD-FMK or Z-LEHD-FMK in the presence or absence of TT101 under the conditions described above. After 24 hours, cytoplasmic DNA fragmentation was detected by using a DNA cell death detection ELISA^{PLUS} kit (Roche Diagnostics) in accordance with the manufacturer's instructions.

Effect of rofecoxib on DNA fragmentation induced by TT101

Human synovial fibroblasts in 96-well culture plates (2×10^4 cells/well) were pretreated or not pretreated with rofecoxib (1 μM) and then were incubated with TT101 or celecoxib under the conditions described above. After 24 hours, cytoplasmic DNA fragmentation was assessed by using a DNA cell death detection ELISA^{PLUS} kit (Roche Diagnostics) in accordance with the manufacturer's instructions.

Western blotting

RA synovial fibroblasts were lysed in Chaps cell extract buffer (50 mM pipes/HCl, pH 6.5, 0.1 % (w/v) Chaps, 5 mM dithiothreitol, 2 mM EDTA, 10 μg/mL pepstatin, 20 μg/mL leupeptin and 10 μg/mL aprotinin), and then centrifuged at 14,000 rpm for 30 minutes to remove debris. Subsequently, the protein content of the supernatant was determined by the

BCA protein assay (Pierce Biotechnology) with BSA as the standard, and the protein content of the extracts was adjusted to 1 mg/mL. Then the extracts were subjected to SDS-polyacrylamide gel electrophoresis using 15 % (w/v) acrylamide slab gels under reducing conditions. The proteins thus separated were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes (Millipore Corporation, Bedford, MA) with a semidry blotter (Atto Technology, Inc., Tokyo, Japan). After the membranes had been blocked in 10 mM TBS containing 0.1 % Tween-20 (TBS-T) and 5 % skim milk for 1 hour at room temperature, rabbit anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit anti-BID polyclonal antibody (Cell signaling technology, Inc., Beverly, MA) was applied for 18 hours at 4 °C. Then the membranes were washed with TBS-T, and incubation with the secondary antibody (HRP-conjugated goat anti-rabbit antibody at a dilution of 1:10,000 in TBS-T) was performed for 1 hour. After further washing with TBS-T, the protein bands were detected by using an enhanced chemiluminescence Western blot analysis system.

Assay of PGE_2 production

RA synovial fibroblasts were incubated in 24-well plates (5×10^5 cells/well) with IL-1β (1 ng/mL) for 18 hours washed with PBS, Then, and exposed to the test drugs for one hour under the conditions described above. Then arachidonic acid (3 μM) (Cayman Chemical Company, Ann Arbor, MI) was added to the medium. After incubation for 30 minutes, the culture medium was harvested using a syringe and filtered through a 0.22 μm filter (Millipore Corporation). The PGE_2 concentration in the medium was measured using an ELISA kit (Cayman Chemical Company) in accordance with the manufacturer's instructions. Each measurement was performed in triplicate.

RESULTS

Effect on cell proliferation

To determine whether TT101 and TT201 had an inhibitory effect on the proliferation of RA or OA synovial fibroblasts, we first examined the influence of these drugs on cell proliferation (DNA synthesis) by measuring the nuclear incorporation of BrdU (Figure 1). TT101 inhibited the proliferation of both RA synovial fibroblasts (panel A) and OA synovial fibroblasts (panel B) in a concentration-dependent manner. TT201, celecoxib, and SC-236 also inhibited cell proliferation, but their effects were weaker than that of TT101. Rofecoxib had no effect on cell proliferation up to a concentration of 100 μM.

Effect on cell viability

To determine whether TT101 and TT201 had an influence on the viability of RA synovial fibroblasts and OA synovial fibroblasts, we evaluated cell viability using the WST-1 assay (Figure 2). TT101 caused a marked decrease of the cell viability of both RA synovial fibroblasts (panel A) and OA synovial fibroblasts (panel B) in a concentration-dependent manner. The order of potency of the effect of each drug on cell viability was as follows; TT101 > celecoxib = SC-236 > TT201 (for both types of cells). Rofecoxib had no effect at all on cell viability.

Effect on DNA fragmentation

TT101 induced DNA fragmentation, the hallmark of apoptosis, in both RA synovial fibroblasts (Figure 3, panel A) and OA synovial fibroblasts (panel B), and its effect was more potent than that of celecoxib or SC-236. TT201 also induced DNA fragmentation, but was weaker than celecoxib. In contrast, rofecoxib did not induce any DNA fragmentation.

The cell morphology was examined under a light microscope after exposure to TT101, (Figure 4). When the cells were incubated with TT101 (7 μM) for 24 hours (panels C and D) distinctive morphological changes were observed, such as rounding and shrinkage,

and the cells became detached from their neighbors.

Effects of TT101 and caspase inhibitors

Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis, so we investigated whether TT101 induced the activation of caspase-3 (a terminal enzyme in the apoptotic pathway). As shown in Figure 5 (panel A), incubation of RA synovial fibroblasts with 7 μM TT101 for 24 hours induced the activation of caspase-3, while this activation was completely blocked by incubation with Z-IETD-FMK or Z-LEHD-FMK (inhibitors of caspase-8 and -9, respectively). Next, we examined the effects of these caspase inhibitors on the TT101 induced-apoptosis (Figure 5, panel B). Induction of DNA fragmentation in RA synovial fibroblasts by TT101 was suppressed by Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK (inhibitors of caspase-3, -8 and -9, respectively) in a concentration-dependent manner.

Western Blot Analysis of Bcl-2 and BID

We examined the effects of the test drugs on the expression of Bcl-2, which is linked to inhibition of apoptosis, by RA synovial fibroblasts. Western blotting demonstrated expression of Bcl-2 by RA synovial fibroblasts, and little change in the level of expression was seen after treatment with any of the COX-2 inhibitors (Figure 6, upper). We also studied the effects of the each drug on cleavage of BID (a substrate of caspase-8) in RA synovial fibroblasts, since truncated BID (tBID, 15 kDa) activates the mitochondrial pro-apoptotic pathway. Full length BID was expressed in RA synovial fibroblasts, but none of the test drugs caused cleavage of BID under the conditions tested (Figure 6, lower).

Effect of rofecoxib pretreatment on DNA fragmentation induced by TT101

To investigate whether the interaction between TT101 and the COX-2 was related to the induction of apoptosis, we studied the effect of pretreatment with another COX-2 inhibitor (rofecoxib) on TT101-induced apoptosis. One hour before addition of TT101, rofecoxib was

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added to cultures of human synovial fibroblasts at a concentration that was sufficient to inhibit COX-2 and then DNA fragmentation was assessed as described above. As shown in Figure 7, TT101 induced the same extent of apoptosis after pretreatment with rofecoxib as that seen in the absence of COX-2 inhibition by rofecoxib, this was true for both in RA synovial fibroblasts (panel A) and OA synovial fibroblasts (panel B).

Effect on production of PGE_2

We investigated the effect of each test drug on the production of PGE_2 by RA synovial fibroblasts. As shown in Figure 8, all of the drugs suppressed the production of PGE_2 (stimulated by the addition 3 μM of arachidonic acid) in a concentration-dependent manner, but TT101 had the weakest suppressive effect on PGE_2 production.

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DISCUSSION

TT101, a novel celecoxib derivative, was shown to be a powerful inducer of apoptosis in both RA synovial fibroblasts and OA synovial fibroblasts. The potency of the inhibitory effect of TT101 on cell proliferation, evaluated in terms of the 50 % effective concentration, was about 5-fold stronger than that of celecoxib for RA synovial fibroblasts and 15-fold stronger for OA synovial fibroblasts. On the other hand, the potency of TT101 for inhibiting COX-2 (50 % inhibitory concentration) was about 70-fold weaker than that of celecoxib. Thus, the pro-apoptotic activity of TT101 was far stronger than that of celecoxib, while its COX-2 inhibitory activity was much weaker. The pro-apoptotic activity of TT101 was not altered by binding of rofecoxib with COX-2 after pretreatment of cultured cells with adequate concentrations of rofecoxib (Figure 7). This allows us to rule out the possibility that binding of TT101 to COX-2 alters the structure of the enzyme and thus triggers apoptosis, suggesting that the mechanism of apoptosis induction by TT101 (as well as celecoxib) involves neither COX-2 inhibition nor structural changes to the COX-2 molecule.

We examined the pro-apoptotic effect of TT101 on U937, a human monocyte cell line. TT101 as well as celecoxib induced cell death in U937 almost the same potency as those in synovial cells (date not shown). A strong pro-apoptotic activity of TT101 on human synovial cells might be one of the therapeutic approaches for rheumatoid arthritis (Nishioka et al., 1998), however, a major improvement of TT101 itself and/or a proper targeting technique for the drug will be necessary for the clinical application.

In our previous study of adenocarcinoma cells, phosphorylation of Akt was only induced by celecoxib among the selective COX-2 inhibitors tested (Yamazaki et al., 2002). Accordingly, the results of the present study using synovial fibroblast and OA synovial fibroblast differ from those of previous studies conducted using tumor cell lines. Zhu et al. (Zhu et al., 2004) synthesized various derivatives of celecoxib and examined each derivative

for induction of the apoptosis of PC-3 human prostate cancer cells. They demonstrated that stronger inhibition of 3-phosphoinositide-dependent kinase-1 (PDK-1), upstream kinase of Akt activation, was associated with stronger induction of apoptosis by celecoxib analogs. In the present study, however, phosphorylated Akt was detected in RA synovial fibroblasts, but it was unaffected by treatment with TT101 or celecoxib (data not shown). The different types of cells used in the two studies may explain the differences in the pro-apoptotic effects of celecoxib.

Apoptosis can be induced by internal (mitochondria-dependent) and external (death receptor-dependent) pathways (Baier et al., 2003). In the mitochondria-dependent pathway, cytochrome c and apoptotic protease activating factor (Apaf)-1 are released from the mitochondria, and then bind to pro-caspase-9 to produce active caspase-9. In the death receptor-dependent pathway, extracellular death ligands bind to receptors and cause activation of caspase-8. The present study showed that, TT101 activated caspase-3, which is at the end of the caspase cascade. Also, the induction of DNA fragmentation induced by TT101 was suppressed by all of the caspase inhibitors tested (inhibitors of caspases-3, -8 and -9). These findings suggest that the pro-apoptotic activity of TT101 may involve two signal transduction pathways, i.e., both the internal (mitochondria-dependent) pathway and the external (death receptor-dependent) pathway.

The Bcl-2 family is thought to be involved in the regulation of these pathways (Tsujimoto and Shimizu, 2000). Bcl-2 prevents various apoptotic mitochondrial changes, including cytochrome c release and loss of the membrane potential (Zamzami et al., 1996). Similar to the observations obtained in our previous study using HT-29 cells (Yamazaki et al., 2002), in which celecoxib did not affect Bcl-2 expression, TT101 did not alter the expression of this anti-apoptotic protein. BID is a specific substrate of caspase-8 involved in the external death receptor-dependent pathway (Luo et al., 1998; and Li et al., 1998). Death

receptor-dependent signals cause cleavage of cytosolic BID to release truncated BID (tBID), which tBID translocates to mitochondria and thus transduces apoptotic signals from the cytoplasmic membrane. Although TT101-induced DNA fragmentation was suppressed by the addition of a caspase-8 inhibitor, we found that BID was not degraded by TT101. It seems likely that the mechanisms of mitochondria-mediated apoptosis are not only regulated by Bcl-2 and BID, but also by various other factors, including Bad, Bim, and Bcl-X_L (Tsujimoto and Shimizu, 2000). We cannot rule out the involvement of such mechanisms in the induction of apoptosis by TT101. Further studies including *in vitro* experiments to investigate the mechanisms of TT101-induced apoptosis and *in vivo* experiments to examine the anti-rheumatic activity using animal arthritis models remain to be conducted.

We modified the sulfonamide group of celecoxib to an N-(2-aminoethyl)-sulfonamide group when developing TT101, while the tolyl group in the terminal aromatic ring of celecoxib was modified to an aminophenyl group to create TT201. In the case of SC-236, this region was changed to a chlorophenyl group. The structures of these celecoxib derivatives and rofecoxib are shown in Figure 9. While TT101 had a strong pro-apoptotic effect, the pro-apoptotic activity of TT201 for RA and OA synovial fibroblasts was much weaker than that of celecoxib. This result suggests that the methyl group of celecoxib may be essential for a pro-apoptotic effect on synovial fibroblasts, when the inhibition of PGE_2 production via COX-2 inhibition was evaluated, TT201 was less potent than celecoxib, but the 50 % inhibitory concentration of TT201 was comparable to that of rofecoxib. As suggested by Zhu et al. (Zhu et al., 2004), the basic frame and electron density of TT101 and TT201 may influence their apoptosis-inducing activity and COX-2 inhibitory activity.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Effect of the each drug on the proliferation of synovial fibroblasts obtained from patients with rheumatoid arthritis (RA, panel A) and osteoarthritis (OA, panel B). Cells were incubated with celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles) for 24 hours. Then proliferative activity was estimated from the nuclear incorporation of BrdU, and was expressed as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown.

Figure 2. Effect of each test drug on the viability of synovial fibroblasts obtained from patients with RA (panel A) and OA (panel B). Cells were incubated with celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles) for 24 hours. Then cell viability was determined by the WST-1 assay and expressed as a percentage of the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown.

Figure 3. DNA fragmentation in synovial fibroblasts from RA patients (panel A) and OA patients (panel B). Cells were incubated with celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles) for 24 hours, after which cytoplasmic DNA fragmentation was measured by enzyme immunoassay and expressed relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown.

Figure 4. Morphological changes of the synovial fibroblasts from RA patients (panels A and C) or OA patients (panels B and D) as observed by light microscopy. Cells were incubated for 24 hours without (A, B) or with (C, D) TT101 at a concentration of 7 μM. Bar = 60 μm.

Figure 5. Effect of TT101 on caspase activity in RA synovial fibroblasts (panel A). Cells were cultured for 24 hours in the absence of any of agents, or with 7 μM TT101 alone, 7 μM

TT101 plus Z-IETD-FMK (a caspase-8 inhibitor), or 7 μM TT101 plus Z-LEHD-FMK (a caspase-9 inhibitor), after which caspase activity was detected by using the CaspACE™ assay system (the absorbance was measured at 405 nm). Data are the mean ± S.D. from 3 independent experiments. *, $p < 0.01$ vs. cells treated with TT101 alone. Significance was evaluated by Tukey's multiple comparison test. Effects of TT101 plus a caspase inhibitor on DNA fragmentation in RA synovial fibroblasts (panel B). Cells were incubated with TT101 with/without a caspase inhibitor for 24 h, after which the cytoplasmic DNA fragmentation was measured by enzyme immunoassay and expressed relative to the control value (untreated cells). Data are the mean ± S.D. for triplicate cultures, and representative results from 3 independent experiments are shown. *, $p < 0.01$ vs. cells treated with TT101 alone. Significance was evaluated by Tukey's multiple comparison test.

Figure 6. Effects of TT101 and celecoxib on expression of Bcl-2 and cleavage of BID. RA synovial fibroblasts were cultured for 6 hours without any agents (lane 1) or were incubated with 7 μM TT101 (lane 2), 100 μM TT201 (lane 3), or 40 μM celecoxib (lane 4). Protein extracts were prepared from the cells and subjected to Western blotting using an antibody that detected Bcl-2 (upper) or BID (lower), as described in Methods.

Figure 7. Effect of rofecoxib on TT101-induced DNA fragmentation. RA synovial fibroblasts (panel A) and OA synovial fibroblasts (panel B) were incubated with (continuous line) /without (broken line) 1 μM rofecoxib for 1 hour, and then were incubated in the presence/absence of TT101 (triangles) or celecoxib (diamonds) for 24 hours. Cytoplasmic DNA fragmentation was measured by enzyme immunoassay and expressed relative to the control value (untreated cells). Data are means ± S.D. from triplicate cultures, and representative results from 3 independent experiments are shown.

Figure 8. Effect of the test drugs on the production of prostaglandin (PG) E_2 by RA synovial fibroblasts. Cells were stimulated with IL-1β (1 μg/mL) for 24 hours, washed twice with PBS,

and incubated for 1 hour at 37°C with various concentrations of celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles). Then arachidonic acid (3 μM) was added and incubation was continued for another 30 minutes, after which the PGE_2 level in the culture medium was measured by enzyme immunoassay. Representative results from 3 independent experiments are shown, and values are the mean ± S.D. from triplicate cultures. The PGE_2 level in control cultures was 38.0 ± 5.9 ng/mL.

Figure 9. Chemical structures of the drugs used

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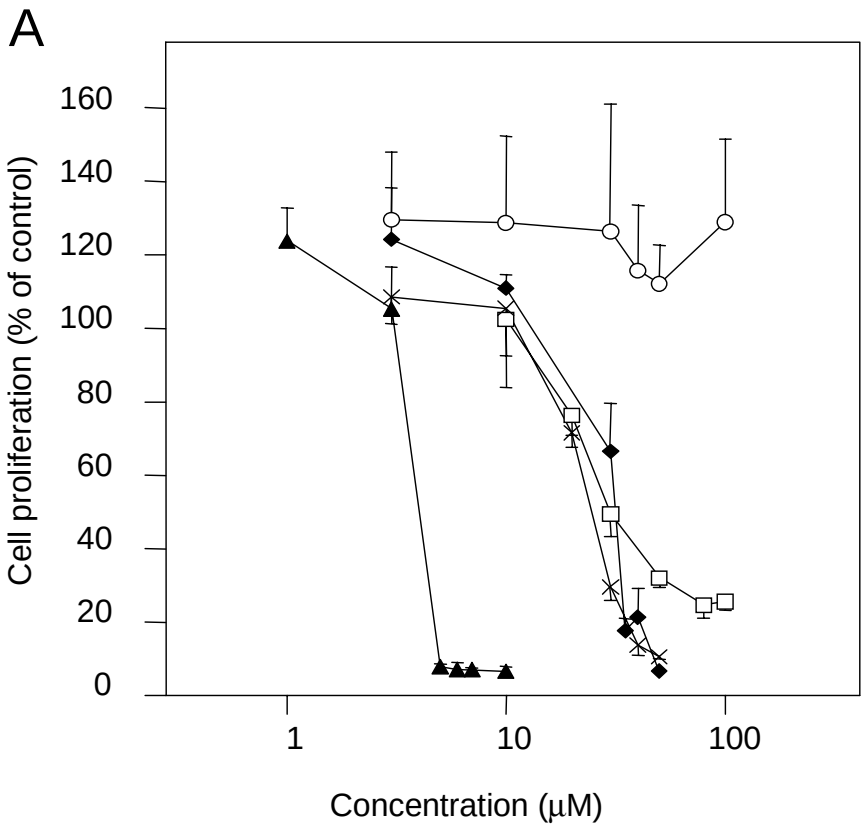
Figure 1



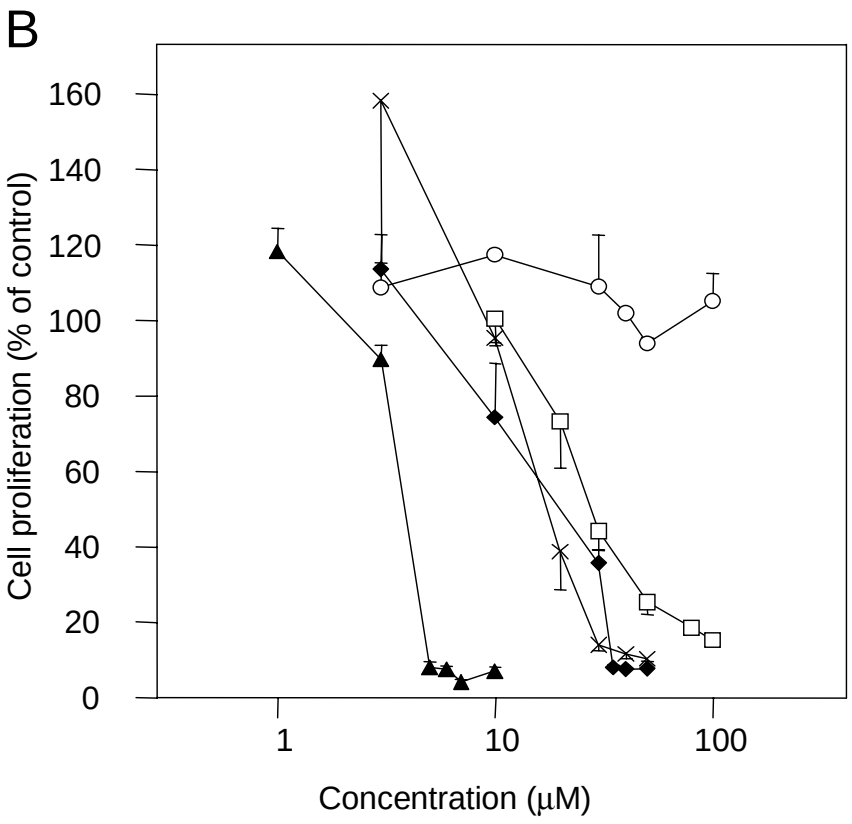


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Figure 2

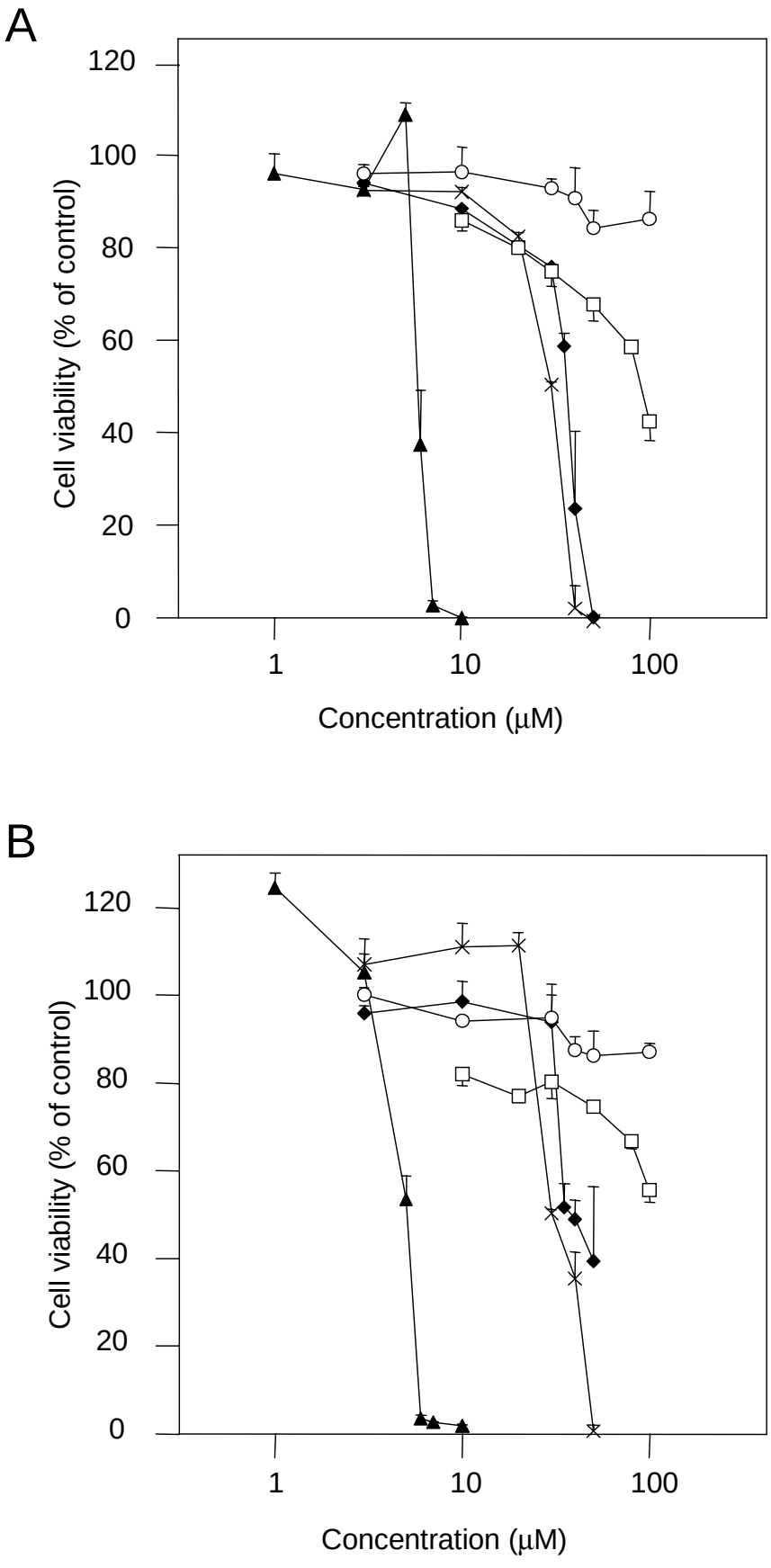

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Figure 3

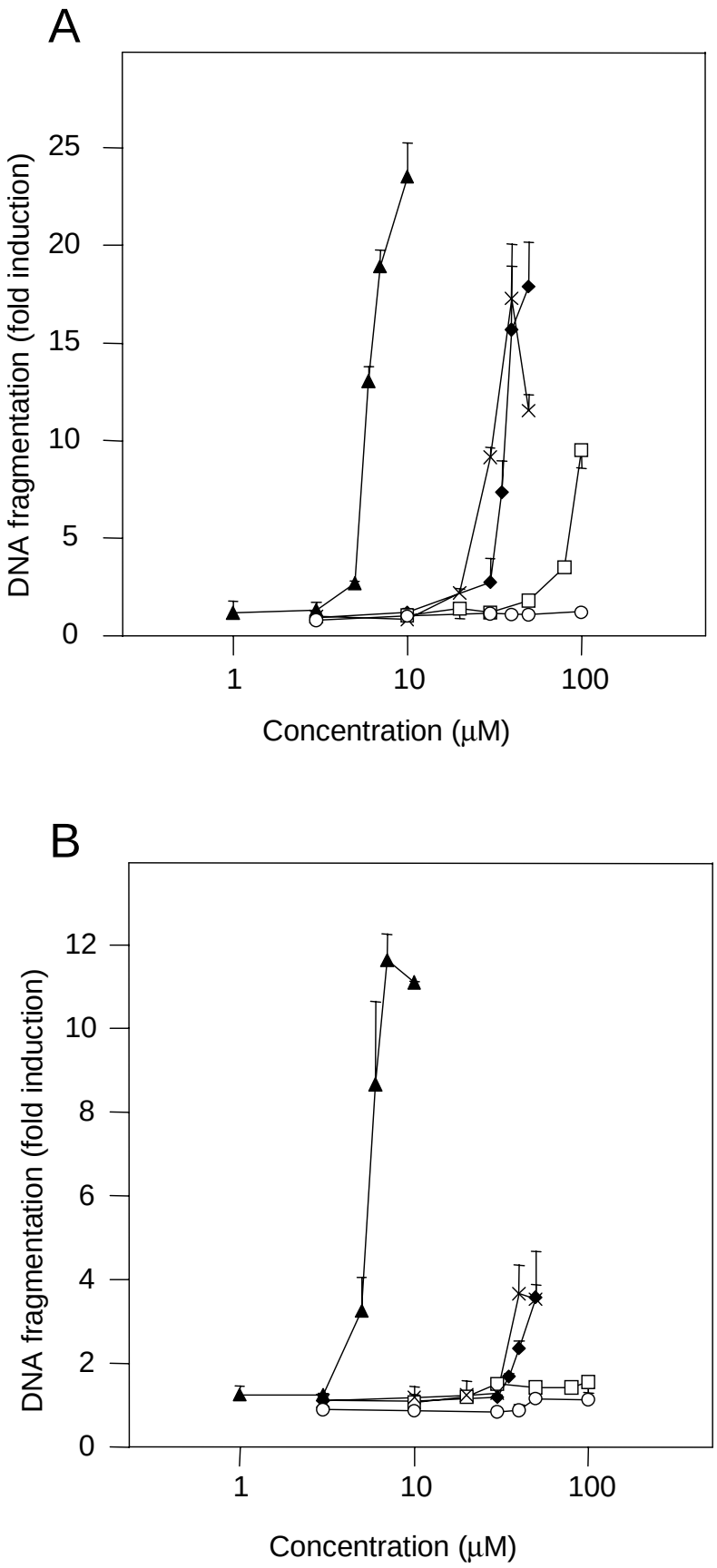

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Figure 4

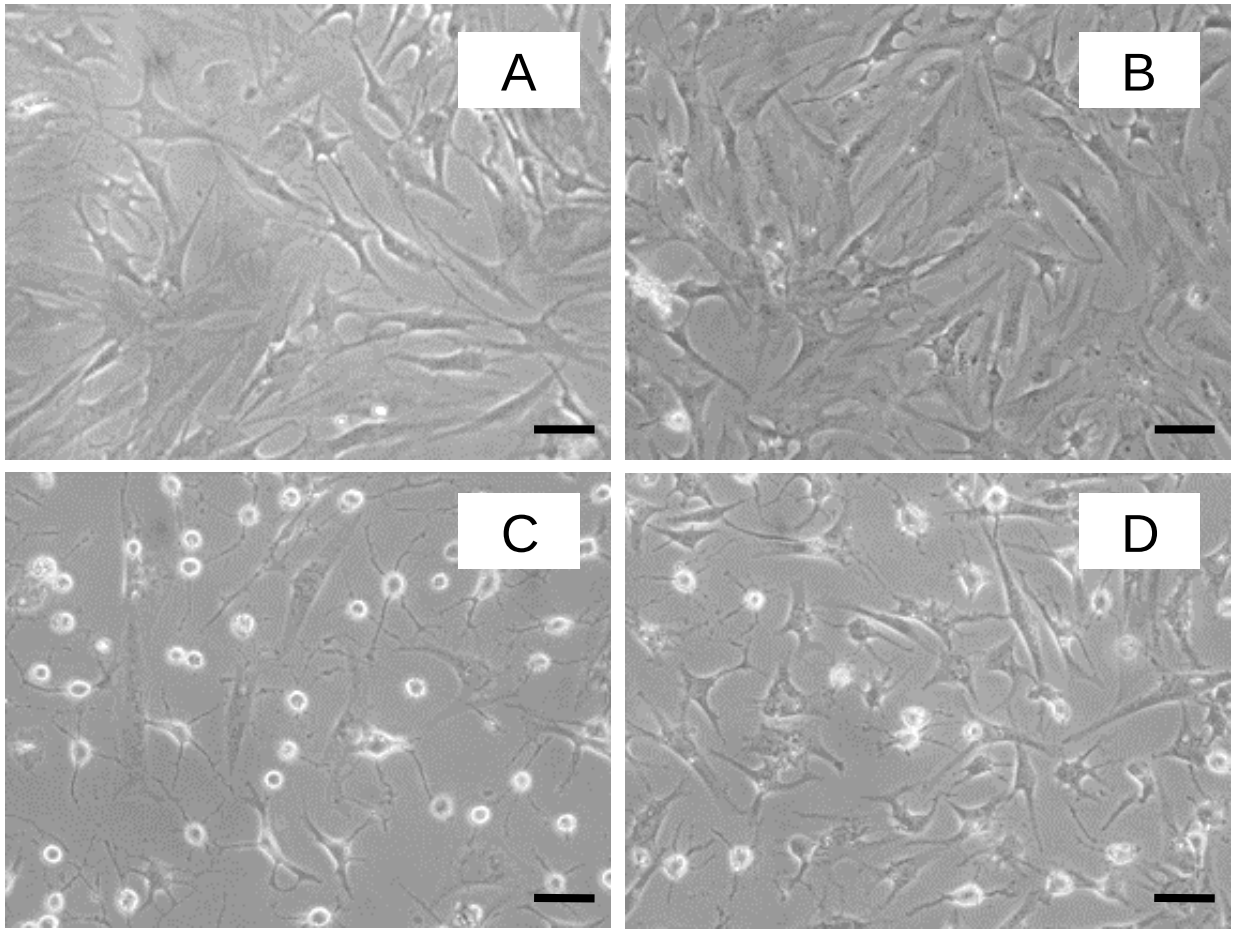

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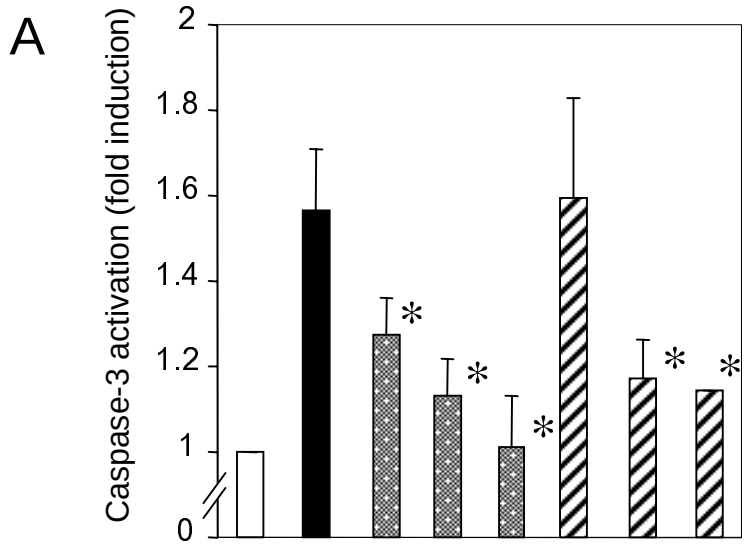
Figure 5



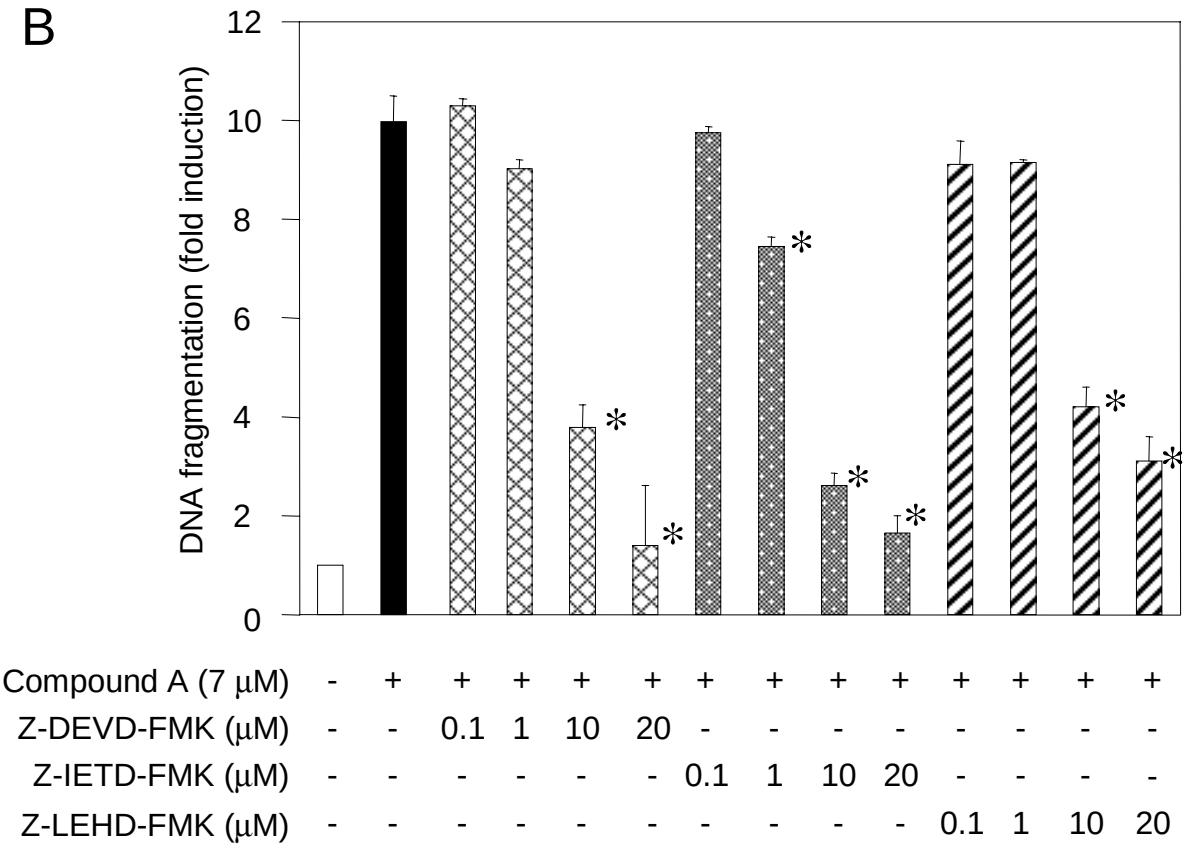


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Figure 6

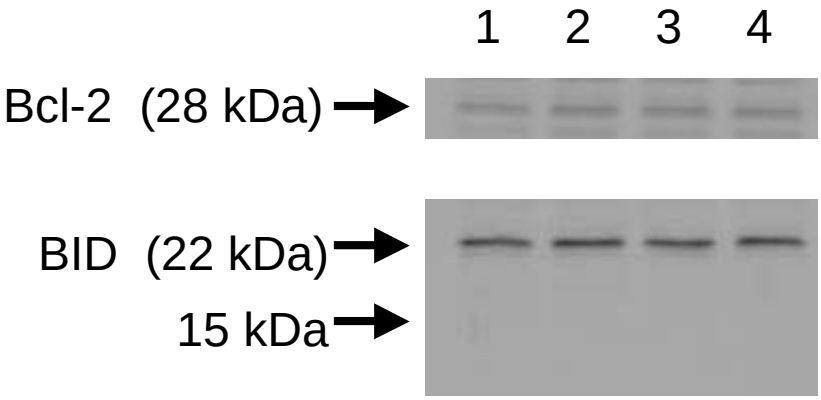

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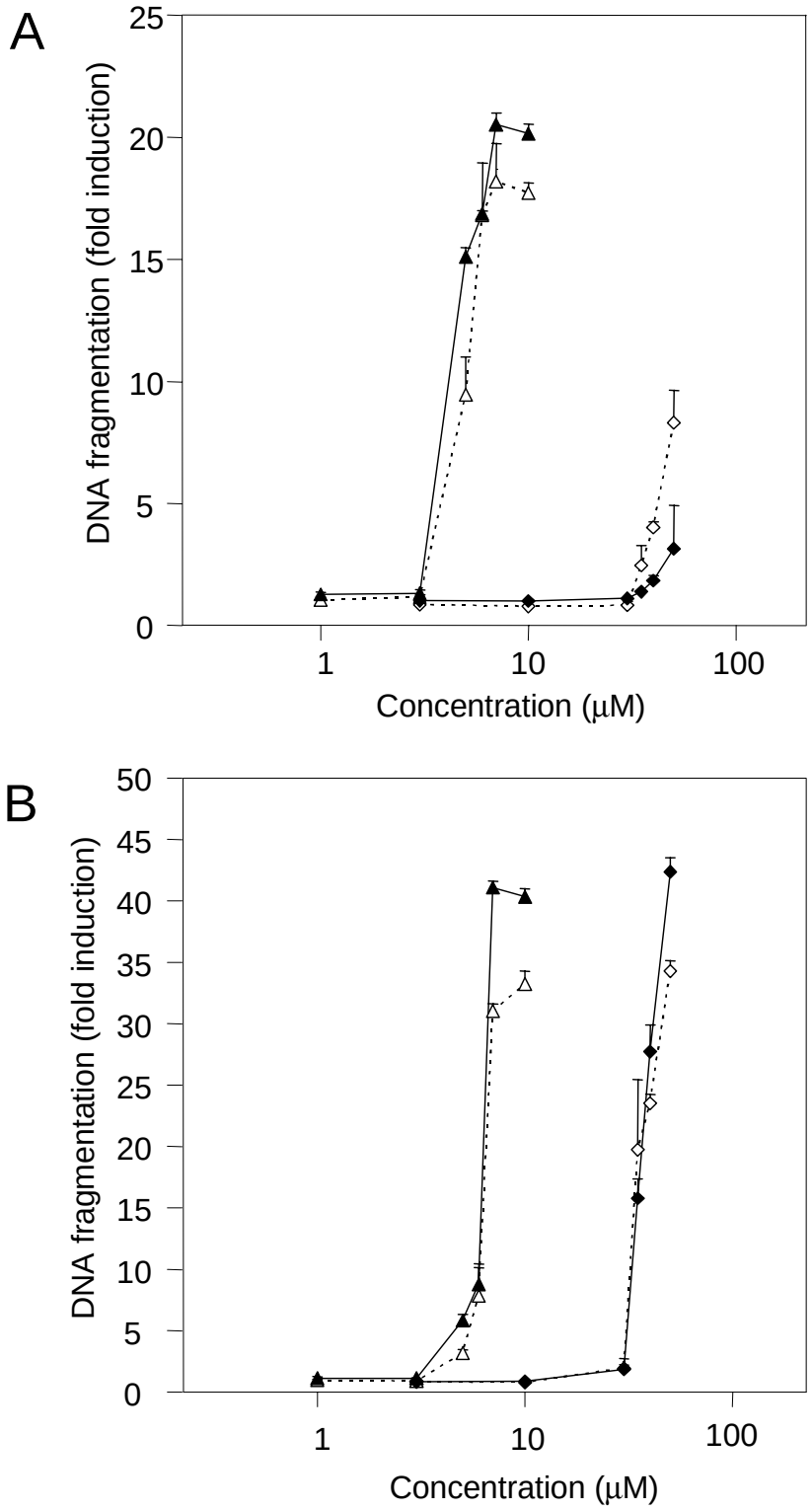
Figure 7



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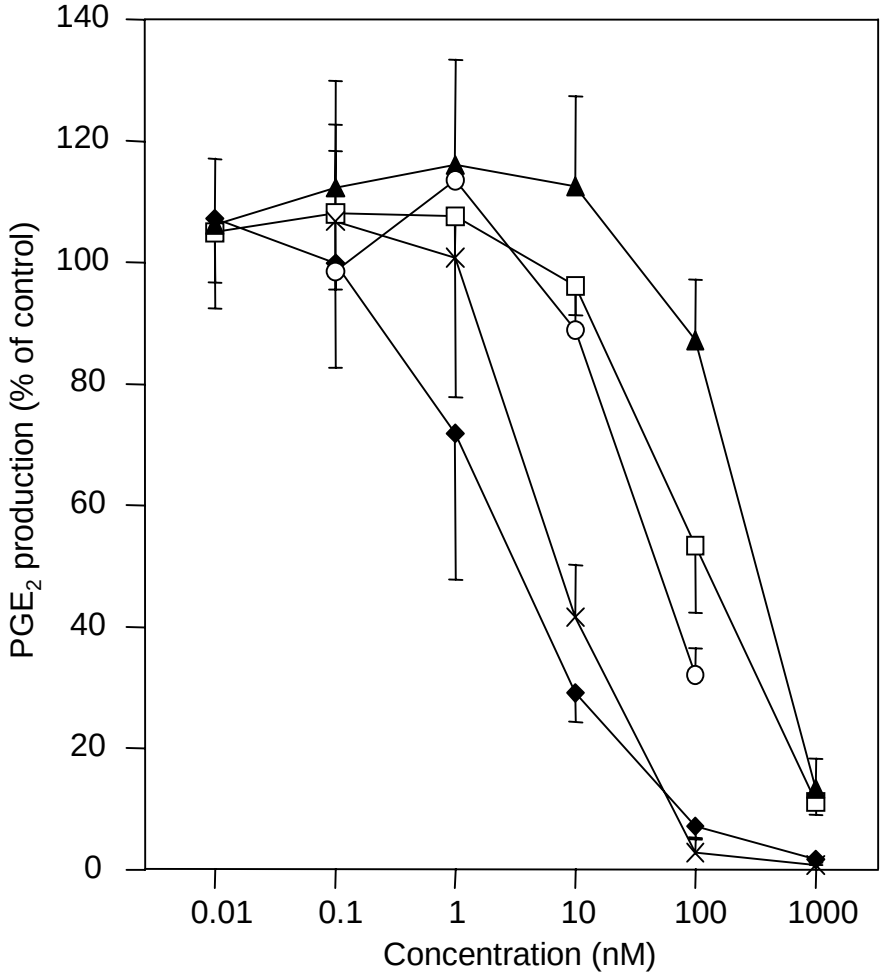

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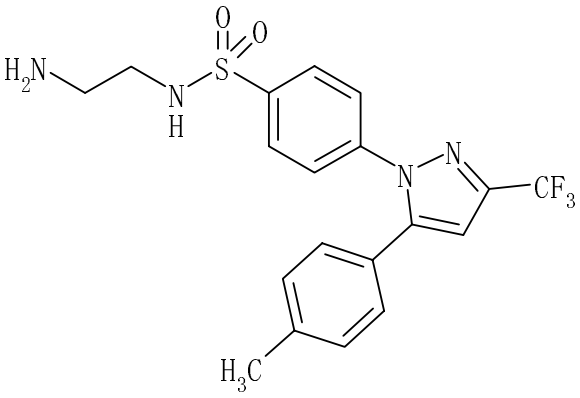
Figure 9



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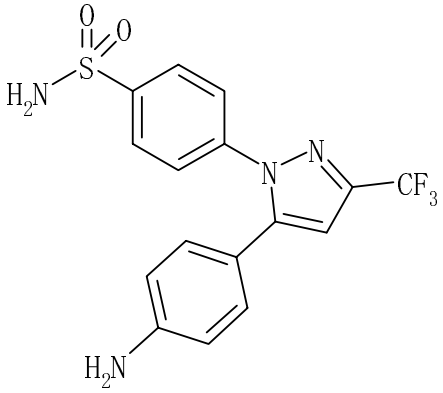

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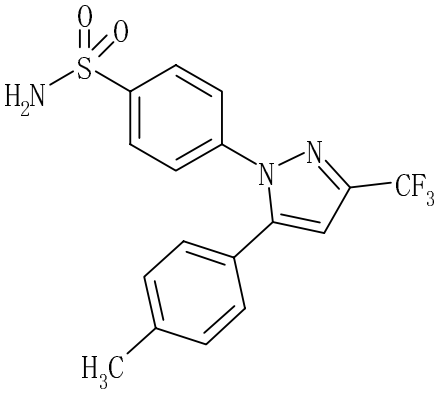

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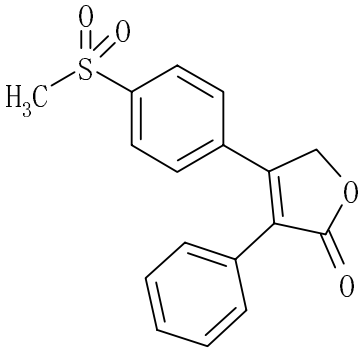

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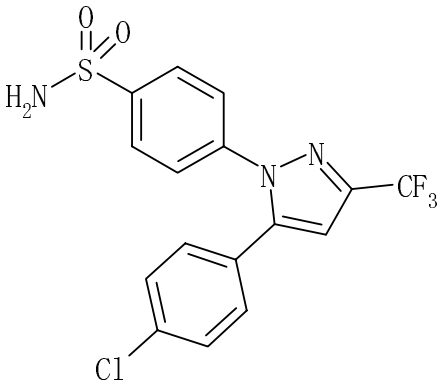

SC236