A Celecoxib Derivative Potently Inhibits Proliferation of Colon Adenocarcinoma Cells by Induction of Apoptosis

NATSUKO KUSUNOKI1, TAKUMI ITO2, NOBUYUKI SAKURAI2, HIROSHI HANDA2 and SHINICHI KAWAI1

Abstract. Background: Celecoxib, a selective cyclooxygenase (COX)-2 inhibitor, has a pro-apoptotic effect on colon adenocarcinoma cells via COX-independent mechanisms. Materials and Methods: The pro-apoptotic effect of N-(2-Aminoethyl)-4-[5-(4-tolyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide (TT101), a new derivative of celecoxib, was investigated on the HT-29 and SW480 colon adenocarcinoma cells. Cell proliferation and viability were assessed by incorporation of 5-bromo-2'-deoxyuridine and by the 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt assay, respectively. Apoptosis was detected by identifying DNA fragmentation. Production of prostaglandin E2 by the HT-29 cells was analyzed. Results: TT101 inhibited the proliferation of HT-29 and SW480 cells by inducing apoptosis more potently than celecoxib in a concentration-dependent manner. The COX-2 inhibitory effect of TT101 was weaker than that of celecoxib. Conclusion: A slight modification of celecoxib enhanced the pro-apoptotic effect on colon adenocarcinoma cells.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have both therapeutic and toxic effects that are mediated by a reduction of prostaglandin (PG) biosynthesis through the inhibition of cyclooxygenase (COX) synthase. Several studies indicated that COX has 2 isoenzymes, which differ with respect to their basal expression, tissue localization, and induction during the inflammatory process (1, 2). Celecoxib is one of the NSAIDs that selectively inhibits COX-2, which is induced by inflammation. Celecoxib is widely used in the treatment of inflammatory diseases, such as rheumatoid arthritis (RA), with the expectation that an anti-inflammatory effect will result from inhibiting the production of PGs like PGE2 through the suppression of COX-2 activity.

Recently, celecoxib was reported to cause a significant reduction in the number of colorectal polyps in patients with familial adenomatous polyposis (FAP) (3) and its antiproliferative effect in animal and cell culture studies has attracted attention. Results of our previous study indicated that celecoxib induced the apoptosis of colon adenocarcinoma cells (4). In addition, several studies conducted by other investigators demonstrated that celecoxib suppressed the proliferation of various cells by inducing apoptosis (5-10), suggesting that the pro-apoptotic action of celecoxib may be useful in the chemoprevention of tumorigenesis (8, 11). These effects may be unique to the drug celecoxib, rather than being a class effect of COX-2 inhibitors.

In our previous in vitro study (4), the concentration of celecoxib required to induce apoptosis of colon adenocarcinoma cells was slightly higher than the blood level reported in healthy individuals (12). Therefore, for adequate pro-apoptotic activity that achieves an anticancer effect, celecoxib may need to be administered at a higher dose than is currently used clinically. We attempted to develop a celecoxib derivative with more potent pro-apoptotic activity than the parent compound.

Materials and Methods

Materials. N-(2-Aminoethyl)-4-[5-(4-tolyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide (TT101) and 4-[5-(4-Aminophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide (TT201) were synthesized (13). 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 (4.6x150 mm), and the mobile phase was
Cell proliferation. The proliferative activity of cultured colon adenocarcinoma cells was estimated from the uptake of 5-bromo-2’-deoxyuridine (BrdU). Cells (1x10⁴ cells/well) were exposed to the test drugs during culture in 96-well plates under the conditions described above. After 24 h, BrdU (10 µM) was added to the medium and the cells were incubated for another 18 h. Then the cells were fixed and nuclear incorporation of BrdU was measured by measuring the nuclear incorporation of BrdU using a DNA cell death detection ELISAPLUS kit (Roche Diagnostics). The results are presented as percentages relative to the value for untreated control cultures.

Effect on cell viability. Colon adenocarcinoma cells were exposed to the test drugs in 96-well culture plates (2x10⁴ cells/well) under the conditions described above. After 24 h, 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)-2H-5-tetrazolio]-1,3-benzensulfonate (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. Colon adenocarcinoma cells were exposed to the test drugs in 96-well culture plates (2x10⁴ cells/well) under the conditions described above. After 24 h, cytoplasmic DNA fragmentation (an indicator of apoptosis) was investigated using a DNA cell death detection ELISAPLUS kit (Roche Diagnostics) in accordance with the manufacturer’s instructions. Each measurement was performed in triplicate and the results are presented as a percentage relative to the value for untreated control cultures. Cell morphology was also examined under a light microscope (BX51, Olympus Optical Co., Ltd., Nagano, Japan) at a magnification of 100x.

Effects of caspase inhibitors on DNA fragmentation induced by TT101. The HT-29 cells growing in 96-well culture plates (2x10⁴ 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 h, cytoplasmic DNA fragmentation was assessed using a DNA cell death detection ELISAPLUS kit (Roche Diagnostics) in accordance with the manufacturer’s instructions.

Assay of PGE₂ production. The HT-29 cells (5x10⁵ cells/well) were pretreated with the test drugs during incubation in 24-well plates containing RPMI 1640 medium with 1% (v/v) FBS at 37°C under an atmosphere of 5% CO₂. After 1 h, calcium ionophore A23187 (0.5 µM; Sigma) was added to the medium and the cells were incubated for another 30 minutes. After incubation, the culture medium was harvested using a syringe and was filtered through a 0.22 µm filter (Millipore Corporation). The PGE₂ level in the culture medium was then measured using an ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Each measurement was performed in triplicate.

Results

Effect on cell proliferation. To determine whether TT101 or TT201 had an inhibitory effect on the proliferation of colon adenocarcinoma cells, the influence of these drugs on cell proliferation (DNA synthesis) was first examined by measuring the nuclear incorporation of BrdU (Figure 1). TT101 inhibited the proliferation of each colon adenocarcinoma cell line 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 or TT201 had an influence on the viability of colon adenocarcinoma cell lines, cell viability was evaluated with the WST-1 assay (Figure 2). TT101 caused a marked
decrease in the viability of each colon adenocarcinoma cell line in a concentration-dependent manner. TT201 always had a weaker effect on cell viability than that of TT101.

**Figure 1.** Effect of each test drug on the proliferation of HT-29 (A) and SW480 (B) cells. The cells were incubated with celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles) for 24 h. The proliferative activity was then estimated from the nuclear incorporation of BrdU and was expressed as a percentage of the control value determined in the untreated cells. Data are the mean±S.D. for triplicate cultures and representative results from three independent experiments are shown.

**Figure 2.** Effect of each test drug on the viability of HT-29 (A) and SW480 (B) cells. The cells were incubated with celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles) for 24 h. Cell viability was then determined by the WST-1 assay and is expressed as a percentage of the control value measured in untreated cells. Data are the mean±S.D. for triplicate cultures, and representative results from three independent experiments are shown.

Effect on DNA fragmentation. TT101 induced DNA fragmentation, the hallmark of apoptosis, in all of the cells tested (Figure 3), and its effect was more potent than that of celecoxib or SC-236. TT201 also induced DNA fragmentation, but its effect was weaker than that of celecoxib. In contrast, rofecoxib did not induce any DNA fragmentation.
Cell morphology was examined by light microscopy to detect any changes after exposure to TT101, (Figure 4). When the cells were incubated with TT101 (10 μM) for 24 h (panels B and D) distinct morphological changes, such as rounding, shrinking, and detachment from the adjacent cells, were observed.

**Effects of TT101 and caspase inhibitors.** Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis, so the effect of caspase inhibitors on TT101 induced-apoptosis was examined (Figure 5). Induction of DNA fragmentation in HT-29 cells by TT101 was suppressed in a concentration-dependent manner after addition of Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK (inhibitors of caspase-3, -8 and -9, respectively).

**Effect on PGE\(_2\) production.** The effect of each test drug was tested on the production of PGE\(_2\) by HT-29 cells. As shown in Figure 6, all of the drugs suppressed PGE\(_2\) production (stimulated by 0.5 μM calcium ionophore A23187) in a concentration-dependent manner, but TT101 had the weakest suppressive effect.

**Discussion**

TT101, a new derivative of celecoxib, was shown to be a powerful inducer of apoptosis in HT-29 and SW480 cells. When the inhibitory effect of TT101 on cell proliferation was evaluated in terms of the 50% effective concentration, it was about 5- to 10-fold stronger than that of celecoxib for these colon adenocarcinoma cell lines. On the other hand, the inhibitory effect of TT101 on COX-2 in HT-29 cells was weaker than that of celecoxib. We recently reported that TT101 has a strong pro-apoptotic effect on RA synovial fibroblasts (13) and the results obtained in the present study were similar to the findings observed in RA synovial fibroblasts.

We examined the pro-apoptotic effect of TT101 on two colon adenocarcinoma cell lines with differing COX-2 expression patterns. Smith et al. (18) reported that SW480 cells did not express COX-2 and we confirmed this in a previous study (4). In the present study, we showed by RT-PCR that HT-29 cells displayed substantial expression of COX-2, while the SW480 cells showed little expression of this enzyme. Both TT101 and celecoxib induced the death of these cells with almost the same potency, indicating that the pro-apoptotic effect of TT101 does not depend on COX-2 expression.

Apoptosis can be induced by internal (mitochondria-dependent) and external (death receptor-dependent) pathways (19). In the mitochondria-dependent pathway, cytochrome c and apoptotic protease activating factor (Apaf)-1 are released from the mitochondria and bind to pro-caspase-9 to produce active caspase-9. In the death receptor-dependent pathway, extracellular death ligands bind to receptors and cause the activation of caspase-8. The present study showed that induction of DNA

Figure 3. DNA fragmentation in HT-29 (A) and SW480 (B) cells. The cells were incubated with celecoxib (closed diamonds), TT101 (closed triangles), TT201 (open squares), SC-236 (crosses), or rofecoxib (open circles) for 24 h, after which cytoplasmic DNA fragmentation was measured by enzyme immunoassay and is expressed relative to the control value (untreated cells). Data are the mean±S.D. for triplicate cultures and representative results from three independent experiments are shown.
Figure 4. Morphological changes of HT-29 cells (panels A and B) and SW480 cells (panel C and D) as observed by light microscopy. The cells were incubated for 24 h without TT101 (A and C) or with TT101 (B and D) at a concentration of 10 μM.

Figure 5. Effects of TT101 plus a caspase inhibitor on DNA fragmentation in HT-29 cells. The cells were incubated with TT101 with/without a caspase inhibitor for 24 h, after which cytoplasmic DNA fragmentation was measured by enzyme immunoassay and is expressed as a percentage of the control value determined in the untreated cells. Data are the mean ± S.D. for triplicate cultures and representative results from three independent experiments are shown. *p<0.01 vs. cells treated with TT101 alone. Significance was evaluated by Tukey’s multiple comparison test.
fragmentation in HT-29 cells 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 strong pro-apoptotic effect of TT101 on human colon adenocarcinoma cells suggests that it might be a possible treatment for cancer, although major improvement of TT101 and/or an appropriate drug targeting technique would be necessary for its clinical application.

In our previous study of adenocarcinoma cells, phosphorylation of Akt was only induced by celecoxib among the selective COX-2 inhibitors tested (4). Zhu et al. (20) synthesized various derivatives of celecoxib and examined whether each of them could induce the apoptosis of PC-3 human prostate cancer cells. They demonstrated that stronger inhibition of 3-phosphoinositide-dependent kinase-1 (PDK-1), a kinase upstream of Akt activation, was associated with greater induction of apoptosis by celecoxib analogs. In the present study, although phosphorylation of Akt was detected in HT-29 cells, its extent was unaffected by treatment with TT101 (data not shown).

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 substituted by an aminophenyl group to create TT201. In the case of SC-236, this tolyl group was changed to a chlorophenyl group. The structures of these celecoxib derivatives and rofecoxib are shown in Figure 7. While TT101 showed a strong pro-apoptotic effect, the pro-apoptotic activity of TT201 for the colon adenocarcinoma cell lines tested in this study was much weaker than that of celecoxib. Such a result suggests that the methyl group of celecoxib may be essential for pro-apoptotic activity against these cells. When the inhibition of PGE2 production via COX-2 blockade was evaluated, TT201 was less potent than celecoxib, but the inhibitory concentration of TT201 was comparable to that of rofecoxib. As suggested by Zhu et al. (20), the basic structure and electron density of celecoxib derivatives may influence their pro-apoptotic and COX-2 inhibitory activities. Further studies on TT101 are needed, including in vitro experiments to investigate the mechanism of its pro-apoptotic activity and in vivo experiments to examine its chemopreventive effect in animal models.

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References


Figure 7. Chemical structures of the drugs tested.

TT101

TT201

celecoxib

rofecoxib

SC-236


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