Differential Combined Effect of COX Inhibitors on Cell Survival Suppressed by Sorafenib in the HepG2 Cell Line

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Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide.1) Several agents that target multiple signaling pathways have recently been entered into clinical trials for HCC patients.2)

Sorafenib, a molecular-targeted drug, is a multi-target oral anti-neoplastic drug that is used as a first-line treatment for patients with advanced Human HCC. An increase in the expression of the cyclooxygenase-2 (COX-2) protein and sequential production of prostaglandin (PG) E2 were previously shown to significantly enhance carcinogenesis. Although the synergistic and/or additive effects of various COX inhibitors have been demonstrated in HCC, those of a combination of sorafenib and COX inhibitors remain unclear. The aim of the present study was to examine the antitumor effects of a combination of sorafenib and COX inhibitors on HCC HepG2 cells. Various COX inhibitors suppressed HepG2 cell survival, and exhibited a combined effect with sorafenib. However, COX-2 selectivity had little relevance. The co-administration of COX inhibitors and sorafenib increased the frequency of apoptosis. Moreover, the combination of sorafenib and diclofenac significantly increased Bax protein expression levels. The results of the present study indicate that COX inhibitors can be administered in combination with sorafenib for HCC therapy.

Key words sorafenib; cyclooxygenase (COX) inhibitor; HepG2; combined effect; apoptosis; Bax

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide.3) Several agents that target multiple signaling pathways have recently been entered into clinical trials for HCC patients.4)

Sorafenib, a molecular-targeted drug, is a multi-target oral anti-neoplastic drug that is used as a first-line treatment for patients with advanced human HCC.5) Two randomized, controlled trials reported the clinical benefits of single-agent sorafenib in extending overall survival in patients with advanced HCC.5,6)

Dal Lago et al.7) reviewed combination therapies of sorafenib with other anti-neoplastic drugs. The anti-neoplastic activity of sorafenib in combination with chemotherapeutic agents such as doxorubicin, gemcitabine, and cisplatin has been reported in preclinical studies.8) A randomized phase II study showed that progression-free survival in adult patients with advanced HCC was significantly longer with a combination of doxorubicin and sorafenib than with doxorubicin alone or placebo.7) A multicenter, randomized, placebo-controlled phase III study reported that the time to tumor progression and overall survival time in adult patients with advanced HCC were significantly better with sorafenib than with placebo,4 and diarrhea, fatigue, and hand–foot skin reaction (HFSR) were noted as the most prevalent grade III toxicities. Lee et al. investigated the effects of S-1, the fourth generation oral fluoropyrimidine, at 4 different dose levels with a fixed dose of sorafenib on advanced HCC in a phase I study.8) When the dose of sorafenib increased, the frequency of adverse events also increased. In addition, the prevalence of drug-related adverse events of any grade was higher in patients treated with sorafenib plus doxorubicin than in patients treated with doxorubicin alone and included HFSR, nausea, vomiting, diarrhea, and leukopenia.9) Therefore, the combination of anti-neoplastic agents increased the prevalence of adverse events.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for inflammation, and their potential roles as cancer chemo-preventive agents have been subject to intensive studies.10–14) NSAIDs inhibit cyclooxygenase (COX), which consequently reduces inflammation. The increased expression of the COX-2 protein and sequential production of prostaglandin (PG) E2 have also been shown to significantly enhance carcinogenesis and inflammatory reactions by upregulating epidermal growth factor receptor (EGFR), phosphatidylinositol-3 kinase (PI3K), and extracellular signal-regulated kinase (ERK) 1/2 signaling.15) The hepatic stellate cells mainly resulted from the ERK-dependent apoptosis via Bax.16) Apoptosis was shown to be enhanced by Bax.7) Therefore, many COX inhibitors are known to possess anti-neoplastic properties.18–22) Many studies have recently suggested that COX inhibitors combined with anti-neoplastic agents have synergistic and/or additive effects on HCC, non-small cell lung cancer, advanced renal cell carcinoma, advanced pancreatic cancer, ovarian cancer, and breast cancer.18–22) However, whether the combination of sorafenib and COX inhibitors has synergistic and/or additive effects on HCC, similar to various COX inhibitors, remains unclear.23,24) Furthermore, whether COX selectivity is associated with the suppression of cell survival has yet to be elucidated. Therefore, the aim of the present study was to examine the effects of combining sorafenib and COX inhibitors on cell survival in HCC HepG2 cells.

MATERIALS AND METHODS

Reagents The human hepatoma cell line HepG2 was obtained from the RIKAKEN BioResource Center (Ibaraki, Japan). RPMI1640, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Life Technologies (Tokyo, Japan). Celecoxib, a selective COX-2 inhibitor, was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). SC-58125; a selective COX-2 inhibitor, etodolac; a preferential COX-2 inhibitor, diclofenac sodium salt; a non-selective inhibitor of COX-1 and COX-2, sulindac; an inhibitor

Note

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of both COX-1 and COX-2, and SC-560; a selective COX-1 inhibitor were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The MEBCYTO® Apoptosis Kit (Annexin V-FITC Kit) was purchased from Medical & Biological Laboratories Co., Ltd. (Aichi, Japan). Sorafenib was dissolved in dimethyl sulfoxide (DMSO), and COX inhibitors were dissolved in PBS (pH 7.4).

**Cell Culture** Cells were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS at 37°C in a humidified atmosphere containing 5% CO₂.

**Cell Viability Assay** Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (1×10⁵ cells/well) were plated onto 96-well microplates. After the culture medium was changed, cells were treated with sorafenib alone or sorafenib with a COX inhibitor for 48 h. Cell viability was evaluated using the trypan blue exclusion assay. Cell survival was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenase.25) Cells (1×10⁵ cells/well) were treated with sorafenib alone or sorafenib with a COX inhibitor for 48 h at 37°C. The medium (100 μL) was then incubated with 10 μL of 5 mg/mL MTT solution for 4 h at 37°C. After centrifugation at 1500 rpm for 10 min, the culture medium was removed, and 100 μL of DMSO was added to each well to dissolve formazan. Absorbance was measured at 570 nm using a microplate reader (Model 680 microplate reader; Bio-Rad Laboratories, Inc., Japan). The results were expressed as a percentage based on the ratio of the absorbance of treated cells to that of the control (100%).

**Combined Effect** The combined effect of two compounds was evaluated using a theoretical value, which was defined as follows: Theoretical value = 100−[(1−S×C)×100], where S is the ratio of the absorbance of sorafenib-treated cells; C is the ratio of the absorbance of COX inhibitor-treated cells. The combined effect was defined as follows: The cell viability (%) of the actual value was smaller than that of the theoretical value.

**Annexin V/Propidium Iodide (PI) Staining and Flow Cytometric Analysis** Cells were treated with sorafenib alone or sorafenib with a COX inhibitor for 48 h. Early apoptotic cells were then annexed with annexin V-fluorescein isothiocyanate (FITC) (Invitrogen, Tokyo, Japan) combined with PI by flow cytometry. Briefly, cells were plated in 6-well plates at a density of 1×10⁵ cells/cm². Treated and untreated cells were incubated with FITC-conjugated annexin V and PI for 15 min at 20°C. They were immediately analyzed on a flow cytometer (Beckton Dickinson, FACSCalibur) in their staining solution. Data from 10000 cells were recorded on logarithmic scales. Data analysis was performed using Flow Jo software on cells characterized by their forward/side scatter (FSC/SSC) parameters. Samples stained with annexin V-FITC and PI were represented by dot plots of PI versus the intensity of annexin V. Dot plots were divided into four regions as follows. The lower left region included cells that failed to stain with either annexin V or PI and were considered to be undamaged. The lower right region included cells stained with annexin V that were still PI negative and were considered to be early apoptotic cells. The upper right region included cells stained with both annexin V and PI; they were classified as late apoptotic or necrotic cells. The upper left region included cells that were annexin V-negative, but PI-positive and were dead cells.

**Protein Extraction from Cultured HepG2 Cells** Total lysates from cells were extracted and prepared using the PARIS protein according to the manufacturer’s protocol. Cells from 6-well culture plates were lysed by being incubated in ice-cold cell disruption buffer with 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF) for 10 min. The homogenates were centrifuged (12000 rpm for 2 min at 4°C). Protein concentrations were determined using the DC protein assay kit (Bio-Rad).

**Western Blot Analysis of Bax Protein Expression** Equal amounts of protein from each sample were fractionated on 12% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to PVDF membranes (Hybond P, GE Healthcare U.K., Buckinghamshire, U.K.). Blots were incubated with rabbit monoclonal Bax antibodies (diluted 1:100; Biotechnology, Santa Cruz, CA, U.S.A.) and mouse monoclonal anti-α-tubulin antibodies (diluted 1:2000; Sigma-Aldrich, St. Louis, MO, U.S.A.). After being incubated with the corresponding HRP-conjugated secondary antibody, specific signals were visualized by chemiluminescence using the ECL Western blotting detection system (GE Healthcare, U.K.). Images were obtained and quantified using FUJIFILM Luminescent Image Analysis LAS-3000 (FUJIFILM, Tokyo, Japan) and Multi Gauge (v3.0) software. The signal ratio Bax/α-tubulin was calculated for quantitative analysis to normalize for the loading and transfer of artifacts introduced in Western blotting.

**Statistical Analysis** Cell survival data and the frequency of apoptosis were statistically analyzed by a one-way ANOVA and Dunnett’s test. Combined data were statistically analyzed by an unpaired t-test. Western blot assay data were statistically analyzed by ANOVA and Tukey’s test. The significance level was set at p<0.05.

**RESULTS**

**Effects of Sorafenib on HepG2 Survival** HepG2 cells were treated with different concentrations of sorafenib for 48 h. Cell growth inhibition was then evaluated using MTT assays. The proliferation of HepG2 cells was inhibited by sorafenib in a concentration-dependent manner [F(9, 70)=79.877, p<0.01] (Fig. 1). The IC₅₀ value of sorafenib was 4.81 μg/mL.

**Effects of COX Inhibitors on HepG2 Survival** As shown in Fig. 2, cell proliferation was inhibited by (A) the selective COX-2 inhibitor, celecoxib [F(6, 77)=20.194, 0.01< p<0.001] significantly different from the respective controls. Cont.=Control

**Fig. 1. Effects of Sorafenib on Cell Proliferation**

The percentage of viable cells was determined using the MTT assay. Error bars represent the mean±S.D. of eight independent experiments. **p<0.01 significantly different from the respective controls. Cont.=Control
p<0.01], (B) the selective COX-2 inhibitor, SC-58125 \([F(6, 77)=27.796, p<0.01]\), (C) the non-selective inhibitor of COX-1 and COX-2, diclofenac \([F(6, 77)=4.595, p<0.01]\), (D) the preferential COX-2 inhibitor, etodolac \([F(6, 77)=0.276, p=0.95]\), (E) the inhibitor of both COX-1 and COX-2, sulindac \([F(6, 77)=14.908, p<0.01]\), and (F) the selective COX-1 inhibitor, SC-560 \([F(6, 77)=74.388, p<0.01]\). Post hoc comparisons revealed that cell proliferation was significantly lower with the two higher doses (25 and 50 \(\mu\)g/mL) of celecoxib \((p<0.01\), respectively), each dose (1.56–50 \(\mu\)g/mL) of SC-58125 \((p<0.01\), respectively), at the highest doses (50 \(\mu\)g/mL) of diclofenac and sulindac \((p<0.01)\), and at the two higher doses (25 and 50 \(\mu\)g/mL) of SC-560 \((p<0.01\), respectively) than with the control. However, etodolac did not suppress cell proliferation.

The IC\(_{50}\) values of celecoxib, SC-58125, sulindac, and SC-560 were 28.14 \(\mu\)g/mL, 19.33 \(\mu\)g/mL, 41.76 \(\mu\)g/mL, and 27.34 \(\mu\)g/mL, respectively.

**Combined Effects of 50 \(\mu\)g/mL of the COX Inhibitors on the Effects of 5 \(\mu\)g/mL Sorafenib on HepG2 Survival**

As shown in Fig. 3, the actual value of cell viability was significantly inhibited by the combination of sorafenib with celecoxib, diclofenac, etodolac, SC-560, and sulindac. However, SC-58125 did not inhibit cell proliferation. Post hoc comparisons demonstrated that cell proliferation was significantly lower with celecoxib, diclofenac, etodolac, sulindac, and SC-560 \((p<0.01, p<0.01, p<0.05, p<0.01, p<0.01,\) respectively) than the theoretical value.

**Flow Cytometric Analysis** Based on the finding that the combination of sorafenib with a COX inhibitor inhibited cell proliferation, we investigated whether this effect was due to an increase in apoptosis. HepG2 cells were treated for 48h with 5 \(\mu\)g/mL of sorafenib and 50 \(\mu\)g/mL of the COX inhibitors, stained for the expression of annexin V and permeability to PI, and then analyzed by flow cytometry (Fig. 4).

**Western Blot Analysis** Western blot analysis revealed a significant increase in the expression of Bax in the lysates from HepG2 cells treated with a combination of sorafenib and diclofenac. However, the combination of sorafenib and a COX inhibitor, excluding diclofenac, did not increase the expression of Bax (Fig. 5).

**DISCUSSION**

In present study, we clarified that various COX inhibitors suppressed HepG2 cell survival, and exhibited a combined effect with sorafenib. However, COX-2 selectivity had little relevance. In addition, the co-administration of COX inhibitors and sorafenib increased apoptosis. Therefore, the combination of sorafenib and COX inhibitors may be a potential treatment strategy for human HCC.

COX-2 is frequently overexpressed in many cancers including HCC as well as colon, breast, bladder, and prostate cancers.\(^{26–28}\) In addition, the antitumor activity of celecoxib was suggested to be associated with its ability to induce apoptosis in various cancer cells.\(^{29}\) The molecular mechanisms underlying celecoxib-mediated apoptosis have not yet been fully elucidated, although it appears to involve endoplasmic reticulum stress, up-regulation of the CCAAT/enhancer binding protein (C/EBP) homolog protein (CHOP)/growth arrest DNA damage (GADD) 153, increases in Ca\(^{2+}\) levels, and the down-regulation of surviving anti-apoptotic proteins.\(^{30}\) However, since the COX inhibitors used in the present study
exhibited different affinities to COX-2, its selectivity was not related to cell survival.

Various studies have reported that the combination of sorafenib and COX inhibitors has synergistic and/or additive effects on HCC. However, the mechanism responsible remains unclear. The increase in intracellular PGE2 induced by NSAIDs was shown to induce apoptotic cell death, and this was dependent on the expression of the pro-apoptotic protein Bax. On the other hand, sorafenib did not induce Bax. Morisaki et al. reported that celecoxib acted on the phosphorylation of AKT downstream of PI3K rather than via the inhibitory activity of COX-2. Although a high dose of celecoxib inhibited the production of PGE2, low doses (IC_{10}–IC_{30}) alone or in combination with sorafenib did not produce a similar effect, which suggests that the synergy between celecoxib and sorafenib did not extend to the COX-2-dependent production of PGE2 in HCC cells. In addition, the combination of sorafenib and celecoxib negatively influenced the cellular apoptosis system from two different angles; the inhibition of PI3K-AKT as well as RAS/RAF/MEK. Cervello et al. reported that the combination of sorafenib and celecoxib had strong synergistic cytotoxic effects in HepG2 cells. As the mechanisms responsible, they suggested that the combination of sorafenib and celecoxib may synergistically promote the induction of CHOP mRNA and protein. In a previous in vivo study, the synergistic effects of sorafenib and aspirin were shown to prolong median survival in xenograft HCC models. Furthermore, aspirin suppressed the pro-inflammatory and pro-metastatic effects of sorafenib in HCC by up-regulating of HTATIP2, and this may have been mediated by the inhibition of COX-2 expression. Sorafenib and aspirin were shown to synergistically inhibit cell cycle progression in the colon cancer. Combining these two drugs markedly increased apoptosis via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, the activation of which decreased in levels of the proteins FLIP and Mcl-1 such as anti-apoptotic protein. In the present study, the combination of sorafenib and diclofenac significantly increased the expression of Bax whereas the combination of sorafenib and a COX inhibitor, excluding diclofenac, did not. Diclofenac was previously shown to induce the expression of Bax following hepatocyte injury. COX inhibitors are known to have other antitumor effects in addition to the inhibition of COX. The synergism observed between celecoxib and SC-560 was found to play a potential role in the chemoprevention of ovarian cancer growth. The other mechanisms of celecoxib and sulindac, excluding the inhibition of COX, included the inhibition of angiogenesis at different checkpoints including PI3K/phosphatase and tensin homolog deleted from chromosome 10 (PTEN)/AKT signaling, canonical Wnt/β-catenin signaling, regulation of matrix metalloproteinase (MMP) activation, and inhibition of the inflammatory response by the suppression of nitric oxide (NO) production. The transcriptional activation of GADD45α and its downstream mitogen-activated protein kinase (MAPK)/c-Jun-N-terminal kinase (JNK) pathway as well as increased levels of the caspase-3 precursor protein have been reported in diclofenac-treated acute myeloid leukemia cells. SC-560 was
also shown to increase phosphorylated ERK1/2 levels,45) and decreased the expression of survivin.46) BAX increased only with the combination of sorafenib and diclofenac, suggesting that the HepG2 cell death induced by sorafenib and COX inhibitors was mediated by different pathways. In the future, the involvements of poly(ADP-ribose) polymerase (PARP), caspase activity, and mitochondrial pathways will be examined, in addition to clarifying the mechanism of action of the combinations of sorafenib and COX inhibitors will be revealed.

However, anti-neoplastic agents are known to induce adverse events. Sorafenib has been reported to cause adverse events such as hypertension (HT), diarrhea, and gastrointestinal perforation,47,48) and also cutaneous side effects, including HFSR (palmar-plantar dysesthesia; acral erythema) and rash. In vitro and in vivo evidence has demonstrated that HT occurs as a direct result of the pharmacological activity of vascular endothelial growth factor (VEGF) inhibitors.49) A recent study reported that sorafenib-induced HFSR was directly related to the cumulative sorafenib dose, the development of HT and HFSR was simultaneous, and HFSR was more prevalent in inpatients treated with sorafenib targeting the VEGF receptor and VEGF growth factor.50) Therefore, sorafenib was associated with a significantly higher risk of all-grade HT and HFSR in patients with cancer. In the present study, the combination of sorafenib with celecoxib, diclofenac, and SC-560 exhibited a synergistic effect. Celecoxib and diclofenac involved VEGF signaling,45,52) whereas SC-560 did not.53) Therefore, from the point of view of a decrease in the prevalence of adverse events such as HT and HFSR, SC-560 is recommended in combination with sorafenib for the treatment of HCC. Furthermore, the QOL was considered to be improved in these patients.

An in vitro study reported that the targeted inhibition of aurora kinases by PHA-739358 may offer a promising new approach for the treatment of patients with advanced HCC.54) Combined therapy with anti-HCC molecular-targeted therapy and immunotherapy targeting the activation of natural killer (NK) cells may improve antitumor effects against unresectable HCC and the prognosis of patients with HCC.55) Based on these findings and the results of the present study, our aim is to establish the best treatment for advanced HCC in the future.

In conclusion, the results of the present study indicate that COX inhibitors may become a concomitant drug of sorafenib in HCC therapy.
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