

COX-2 inhibitors sensitize human hepatocellular carcinoma cells to TRAIL-induced apoptosis

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Received January 31, 2006; Accepted March 27, 2006

Abstract. Cyclooxygenase (COX)-2 is upregulated in a variety of human cancers, including in hepatocellular carcinoma (HCC), whereas it is undetectable in most normal tissue. Evidence suggests that COX-2 is likely to be involved in hepatocarcinogenesis and, thus, COX-2 may be involved in an early process in carcinogenesis, dedifferentiation. To address this possibility, we investigated the effect of COX-2 inhibitors on TNF-related apoptosis, inducing ligand (TRAIL) sensitivity and its molecular mechanisms, with special attention to anti-apoptotic proteins. We used the highly selective COX-2 inhibitors, NS398 and CAY10404. We also used the MTT assay and cytological analysis of DAPI-stained DNA to assess viability and apoptosis in two HCC cells (SK-Hep1 and HLE). In order to ask what led to increased sensitivity to TRAIL in HCC cells, cell surface expression of TRAIL and TRAIL-receptors was investigated using flow cytometry analysis. Expression of survivin, X-chromosome-linked IAP (XIAP), Bcl-xL, AKT and phospho-AKT was also investigated using immunoblotting. COX-2 inhibitors resulted in a concentration-dependent decrease in cell viability in the two HCC cell lines tested. Subtoxic levels of COX-2 inhibitors did not significantly augment TNF α -induced apoptosis but did dramatically enhance TRAIL-induced apoptosis in both cell lines. TRAIL receptor 2/death receptor 5 (TRAIL-R2/DR5) expression was significantly up-regulated in SK-Hep1 and HLE cells. TRAIL receptor 1/death receptor 4 (TRAIL-R1/DR4) expression was up-regulated only in SK-Hep1. Expression of survivin and Bcl-xL was down-regulated in SK-Hep1 and HLE cells in the presence of CAY10404 but XIAP was not affected. Expression of survivin, Bcl-xL and XIAP was down-regulated in SK-Hep1 cells in the presence of NS398. Survivin expression was also down-regulated in the presence of NS398 in HLE cells. Finally, NS398 also

decreased phospho-AKT in SK-Hep1 cells. These results demonstrate that COX-2 inhibitors can induce apoptosis and augment TRAIL sensitivity by up-regulation of TRAIL receptors and down-regulation of both survivin and AKT signaling.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, developing from the basic disease of chronic liver injury (1). In hepatocarcinogenesis, failure of apoptosis is considered to be important for the survival of hepatocytes, which may be more prone to genetic damage and cellular transformation via proliferation than other cell types since hepatocytes are a regenerative cell type. Indeed, most HCC cells show strong resistance to stimuli that induce apoptosis in other cells. Several cellular anti-apoptotic mechanisms are known to contribute to resistance against immunologic cytotoxicity in human HCC cells. These include up-regulation of anti-apoptotic proteins, members of the Bcl-2 family, and inhibitor of apoptosis family members such as X-chromosome-linked IAP (XIAP), survivin and FLICE-inhibitory proteins (FLIPs). Therefore, overcoming apoptotic resistance has become critical for the establishment of effective therapeutic strategies for the treatment of HCC.

Cyclooxygenases (COX) are key enzymes in the conversion of arachidonic acid to prostaglandins and other eicosanoids (2). There are two isoforms of COX; COX-1 and COX-2. COX-1 is constitutively expressed in various types of cells and plays important roles in homeostasis. COX-2 is usually absent under basal conditions but is inducible by various cytokines and growth factors and mitogens (3,4). Interestingly, COX-2 has been shown to be upregulated in a variety of human cancers including colon, gastric, esophagus, pancreas, and breast cancer and in HCCs, despite the fact that COX-2 is undetectable in most normal tissue (5-10). In some cancers, such as colorectal carcinogenesis, COX-2 expression is present throughout carcinogenesis but during hepatocarcinogenesis, COX-2 expression is high in early stages of HCC but low in advanced stages. These findings suggest that COX-2 plays an important role in the early stages of hepatocarcinogenesis but not in advanced stages (11). Thus, COX-2 may be related to HCC dedifferentiation, an early event in hepatocarcinogenesis.

COX-2 induces at least three tumor-related processes. Firstly, it affects angiogenesis via an acceleration of production of both vascular and endothelial growth factor, and of

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Key words: COX-2 inhibitor, hepatocellular carcinoma, TRAIL, TNF

prostaglandins (12). Secondly, it affects anti-apoptosis factors normally mediated by Bcl-2 and protein kinase B signaling (13-16). Thirdly, it has a strong effect on invasiveness via the action of matrix metalloproteinases. Selective COX-2 inhibitors such as NS398 can inhibit growth and induce apoptosis in several cancer cell types, including colon and pancreatic cancer cells. Moreover, a selective COX-2 inhibitor can inhibit growth and induce apoptosis in cultured colon and pancreatic cancer cell lines (17,18). The antitumor effects of COX-2 inhibitors are also enhanced in combination therapy with conventional anticancer agents, radiotherapy, and photodynamic therapy (19-22). These findings might expand the therapeutic potential of COX-2 inhibitors. Nonetheless, the molecular and biochemical pathways responsible for the pro-apoptotic effects of COX-2 inhibitors remain poorly understood (17).

TNF-related apoptosis inducing ligand (TRAIL) is a member of the TNF family and selectively induces apoptosis in a variety of transformed cell lines. However, several tumor cell lines, including HCC and most normal cells, are resistant to TRAIL-mediated apoptosis. This selective effect on cancer cells is the basis of the current enthusiasm for TRAIL as a potential target of novel anticancer therapeutics. Previous studies have suggested that HCC cells might be resistant to TRAIL-mediated apoptosis, despite the fact that they express TRAIL receptors (23). Even if this proves true, it may be possible to sensitize HCC cells to TRAIL-induced apoptosis therefore, the study of how to bring about that sensitization is an important topic to be addressed.

In this study, we investigated the effects of COX-2 inhibitors on TRAIL sensitivity and looked at the molecular mechanisms of the action of COX-2 inhibitors with special attention to anti-apoptotic proteins using the highly selective COX-2 inhibitors, NS398 and 3-(4-methylsulphonylphenyl)-4-phenyl-5-trifluoromethylisoxazole (CAY10404).

Materials and methods

Cell lines. The human HCC cell line, SK-Hep1, was purchased from the American Type Culture Collection (Rockville, MD) and HLE (JCRB 0404) was obtained from the Health Science Research Resources Bank (Osaka, Japan). All cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) with 10% heat inactivated fetal calf serum (Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL) in a humidified atmosphere containing 5% CO₂ at 37°C.

Detection of proliferation. To assess HCC cell viability, the MTT assay [formally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay] was performed. HCC cells were plated at a density of 5x10³ cells per well in 96-well microtiter plates (Corning Glass Works, Corning, NY) and each plate was incubated for 24 h at 37°C in 5% CO₂. The HCC cells were allowed to grow to a high density and then the cells were treated with COX-2 inhibitors CAY10404 or NS398 (Cayman Chemical, MI). The COX-2 inhibitors were added in the presence or absence of TRAIL (Santa Cruz Biotechnology, CA) or TNF α (Genzyme-techno, Cambridge, MA) and the plates were incubated for 24 h.

DAPI staining. Staining with DAPI (4'-6-diamidino-2-phenylindole), a DNA-binding fluorescent dye, was performed as described. Briefly, after treatment with 50 μ M NS398 for 24 h, the cells were washed three times with phosphate-buffered saline (PBS), and fixed first in 4% formaldehyde for 10 min and then in methanol. The cells were stained with 4 μ g/ml DAPI (Roche, Germany) for 10 min and changes in nuclear morphology were observed by fluorescence microscopy.

Flow cytometric analysis. Approximately 1x10⁶ SK-Hep1 cells incubated with 10 ng/ml COX-2 inhibitors (100 μ M CAY10404, 50 μ M NS398) for 48 h were washed with PBS, harvested in the presence of 0.02% EDTA in PBS, and incubated for 30 min on ice with phycoerythrin (PE)-conjugated anti-human TRAIL, isotype PE-conjugated mouse IgG, PE-conjugated anti-human DR4 (TRAIL-R1) and PE-conjugated anti-human DR5 (TRAIL-R2; eBioscience, San Diego, CA) at a concentration of 5 μ g/ml. The cells were analyzed by flow cytometry using a FACScan cytometer and CellQuest software (Becton-Dickinson, Tokyo, Japan).

Immunoblotting. HCC cell extracts were homogenized in lysis buffer (50 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid, 1% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride), and equal amounts of protein from each extract were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to cellulose nitrate membranes (Advantec, Tokyo, Japan).

After blocking with 5% milk in Tris-HCl pH 7.5 with 0.1% Tween-20 at 4°C overnight, the blots were incubated with 5 μ g/ml anti-survivin antibody (Santa Cruz Biotechnology) or 10 μ g/ml anti-survivin antibody pre-absorbed with survivin peptide resin overnight at 4°C. In the same way, the blots were incubated with 5 μ g/ml Anti-XIAP (BD Biosciences, MD), anti-Bcl-xL (BD Biosciences), anti-AKT or anti-phospho-AKT antibody pre-absorbed overnight at 4°C. The immunoblots were washed in Tris-HCl pH 7.5 with 0.1% Tween-20 and probed with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Tokyo, Japan; 1:2,000 diluted with 5% milk in Tris-HCl pH 7.5). After washing, the immunoblots were developed using the electrochemiluminescence system (Amersham, CA).

Results

COX-2 inhibitors induced apoptosis in HCC cells. To investigate the change in viability of HCC cells in response to COX-2 inhibitors, HCC cells were incubated with various concentrations of COX-2 inhibitors for 48 h. Cell viability was assessed using the MTT assay (Fig. 1). Cox-2 inhibitors caused a concentration-dependent decrease in cell viability in the two HCC cell lines tested, SK-hep1 and HLE. In order to ask if COX-2 inhibitors can induce apoptosis in HCC cells, we next looked at DAPI staining 24 h after treatment of SK-Hep1 cells with COX-2 inhibitors (Fig. 4). We found that COX-2 inhibitor-treated cells showed features typical of apoptosis, including nuclear condensation and nuclear fragmentation.

COX-2 inhibitors augmented TRAIL induced apoptosis. We further investigated TRAIL-induced apoptosis in HCC cells.

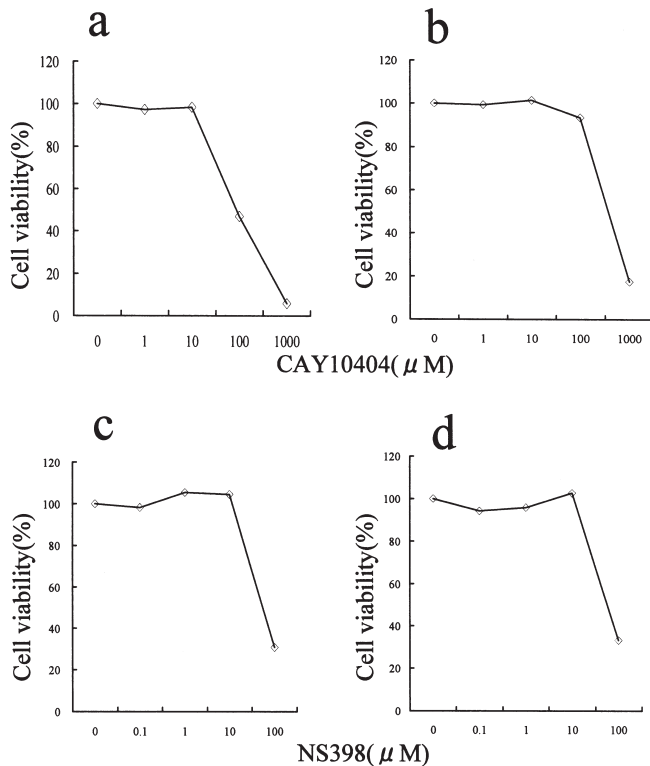


Figure 1. Cox-2 inhibitors induced cell death in human HCC cells. SK-Hep1 and HLE cells were treated with 0-1000 μ M CAY10404 or 0-100 μ M NS398 and analyzed after 24 h for cell viability by the MTT assay. The data shown are representative of three independent experiments. (a) CAY10404 was added to SK-Hep1 cells. (b) CAY10404 was added to HLE cells. (c) NS398 was added to SK-Hep1 cells. (d) NS398 was added to HLE cells.

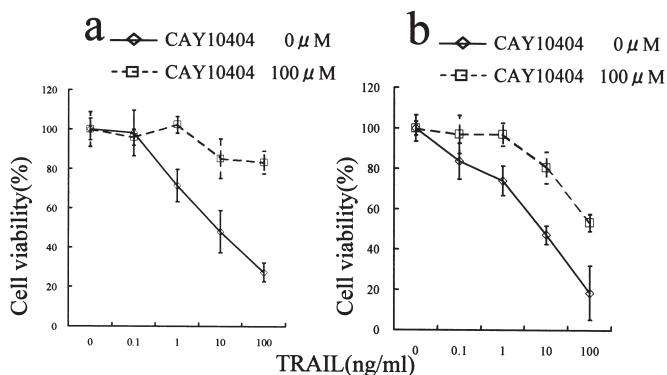


Figure 2. Treatment with both COX-inhibitors and TRAIL augmented cell death in HCC cells. SK-Hep1 and HLE cells were treated with 100 μ M CAY10404 or 0 μ M CAY10404 and 0-100 ng/ml TRAIL or 0-100 ng/ml TNF α and, after 24 h, cell viability was assessed by the MTT assay. The data shown are representative of three independent experiments. 100 μ M CAY10404 or 0 μ M CAY10404 was added. (a) TRAIL was added to SK-Hep1 cells (b) TRAIL was added to HLE cells (c) TNF α was added to SK-Hep1 cell (d) TNF was added to HLE cells.

Unlike many other transformed cell lines, in HCC cells, TRAIL failed to induce apoptosis or even weak cytotoxicity, even at doses of up to 100 ng/ml. However, in the presence of a sub-toxic level of COX-2 inhibitors (100 μ M CAY10404 or 50 μ M NS398), TRAIL-treated cells demonstrated features typical of apoptosis; namely, nuclear condensation and nuclear

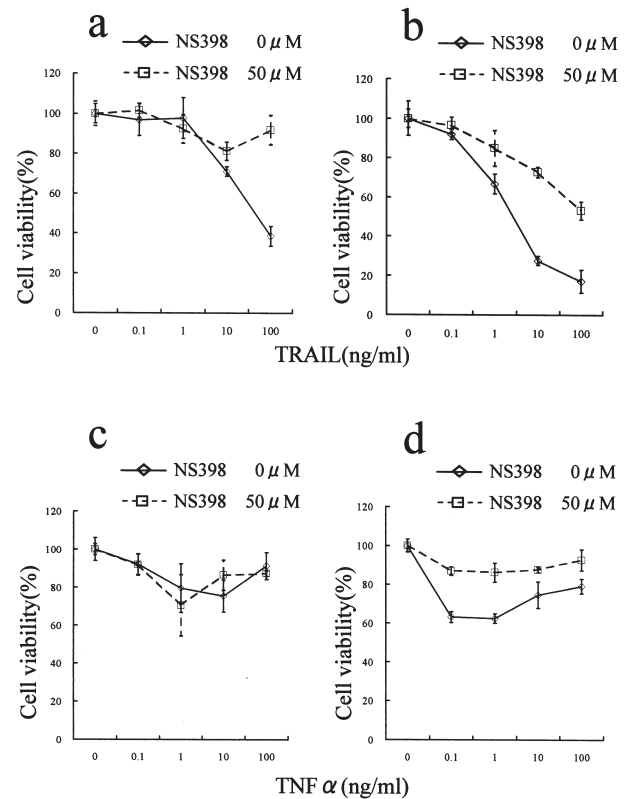


Figure 3. Treatment with both COX-inhibitors and TRAIL augmented cell death in HCC cells. SK-Hep1 and HLE cells were treated with 50 μ M NS398 or 0 μ M NS398 and 0-100 ng/ml TRAIL or 0-100 ng/ml TNF α , and after 24 h, cell viability was assessed by the MTT assay. The data shown are representative of three independent experiments. 50 μ M NS398 or 0 μ M NS398 was added. (a) TRAIL was added to SK-Hep1 cells (b) TRAIL was added to HLE cells (c) TNF α was added to SK-Hep1 cells (d) TNF α was added to HLE cells.

fragmentation (Fig. 4). Treatment with COX-2 inhibitors converted TRAIL-resistant cells into TRAIL-sensitive cells, resulting in TRAIL-induced apoptosis in both HCC cells that was dependent on the concentration of COX-2 inhibitors. Viability of cells 24 h after treatment with TRAIL (100 ng/ml) in the presence of COX-2 inhibitors (100 μ M CAY10404 or 50 μ M NS398) was 27% (CAY10404, SK-Hep1), 18% (CAY10404, HLE), 38% (NS398, SK-Hep1) or 17% (NS398, HLE). Despite this ability to sensitize cells to TRAIL-mediated apoptosis, COX-2 inhibitors did not affect TNF α -induced apoptosis in both cells (Figs. 2 and 3).

COX-2 inhibitors up-regulated TRAIL receptors. In order to examine the mechanisms of increased TRAIL susceptibility in SK-Hep1 and HLE cells, cell surface expression of TRAIL and its receptors was investigated using flow cytometry analysis (Fig. 5). We found that, in SK-Hep1 and HLE cells incubated with COX-2 inhibitors for 48 h, TRAIL-R2/DR5 expression was significantly up-regulated (SK-Hep1 cells) or slightly up-regulated (HLE cells). In addition, TRAIL-R1/DR4 expression was slightly up-regulated in SK-Hep1 cells. However, cell surface expression of TRAIL was not up-regulated in either cell type.

COX-2 inhibitors affected apoptosis regulatory proteins. Bcl-xL, a mitochondrial apoptosis inhibitory protein, and

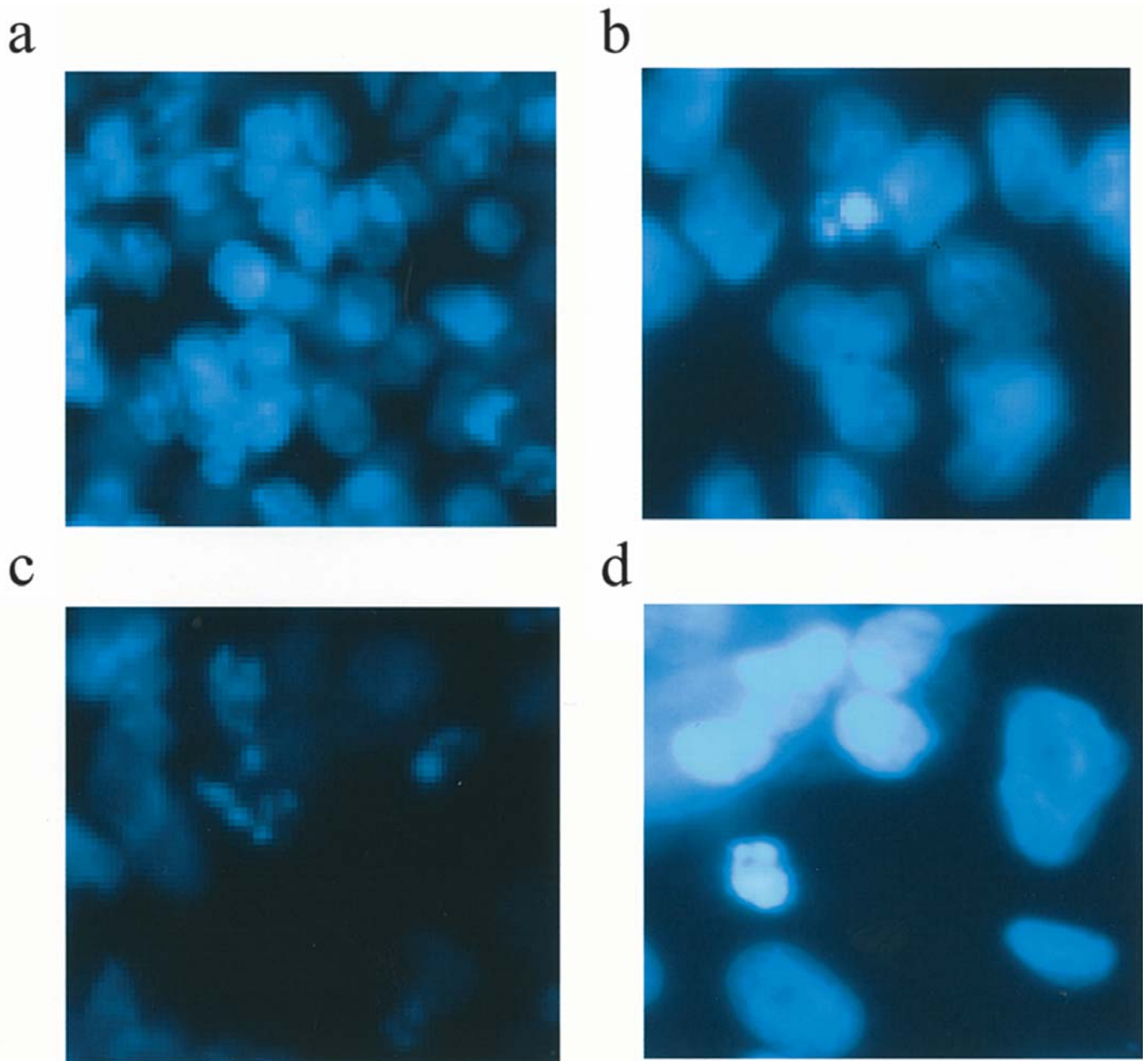


Figure 4. COX-2 and TRAIL inhibitors induced apoptosis in SK-Hep1 cells. Cells were cultured in the (a) absence, or (b) presence of 100 μ M CAY10404, (c) 50 μ M NS398 and (d) 100 μ M CAY10404 and 10 ng/ml TRAIL for 24 h. Cell nuclei were stained with DAPI. Note the features of nuclear condensation and nuclear fragmentation typical of apoptotic cells (b, c and d).

survivin and XIAP, direct inhibitors of cell-death proteases, play major roles in the control of apoptotic pathways. To determine the expression levels of these apoptosis-related proteins in HCC cells, we analyzed their expression by immunoblotting (Figs. 6 and 7). Expression of survivin and Bcl-xL was down-regulated in SK-Hep1 and HLE cells in the presence of CAY10404 but XIAP was not affected. Expression of survivin, Bcl-xL and XIAP was down-regulated in SK-Hep1 cells in the presence of NS398. Survivin expression was also down-regulated in the presence of NS398 in HLE cells.

COX-2 inhibitors modulate the AKT pathway. Finally, we investigated the state of the AKT pathway, since the pathway is important for the balance between cell survival and

apoptosis. To determine whether TI3K/AKT signaling pathways are involved in COX-2 inhibitors-induced apoptosis, we looked at AKT phosphorylation by immunoblotting (Fig. 8). NS398 treatment resulted in a significant and dose-dependent decrease in the level of phosphorylation of AKT in SK-Hep1 cells but did not have an effect on AKT activity in HLE cells.

Discussion

We show that COX-2 inhibitors NS398 and CAY10404 induced apoptosis in two HCC cell types tested and that the effect was concentration dependent. Previous studies have shown that NS398, a selective COX-2 inhibitor, can inhibit growth and induce apoptosis in several other cancer cell lines,

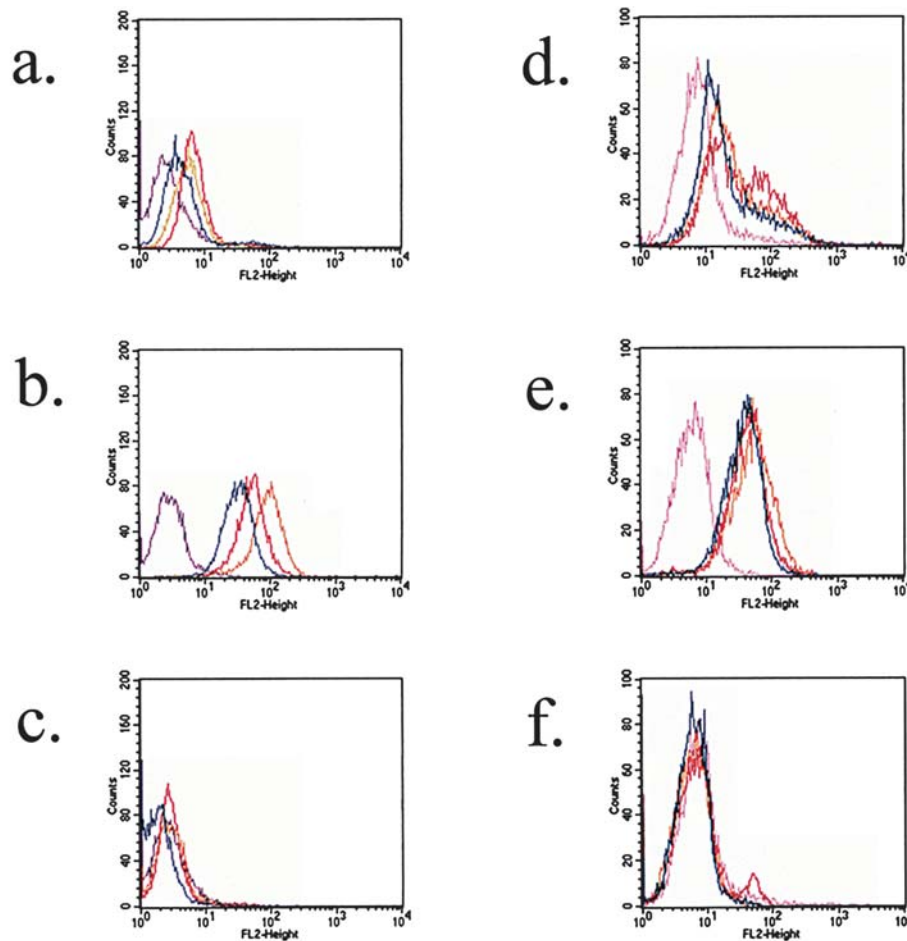


Figure 5. TRAIL-R2/DR5 expression was significantly up-regulated (SK-Hep1 cells) or slightly up-regulated (HLE cells). TRAIL-R1/DR4 expression was slightly up-regulated in SK-Hep1 cells. TRAIL expression was not up-regulated in either cell type. HCC cells were incubated with 100 μ M CAY10404 (red lines) or 50 μ M NS398 (orange lines) for 24 h, and were then stained with PE-anti-human TRAIL-R1/DR4 [(a) SK-Hep1 cells, (d) HLE cells], TRAIL-R2/DR5 [(b) SK-Hep1 cells, (e) HLE cells], or TRAIL [(c) SK-Hep1 cells, (f) HLE cells]. Control cells were not treated (blue lines). Auto-fluorescence and non-specific staining were determined using cells incubated with PE-control mouse IgG (purple lines).

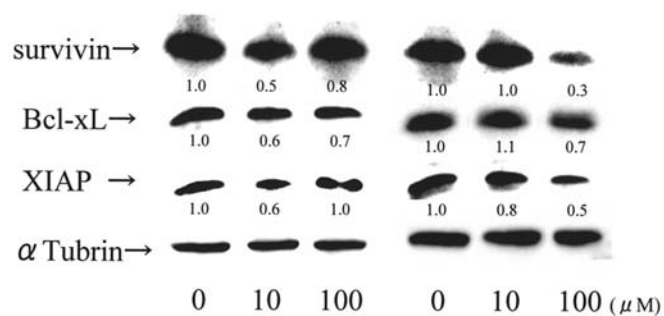


Figure 6. Expression of survivin, Bcl-xL, and XIAP in SK-Hep1 cells after treatment with COX-2 inhibitors. SK-Hep1 cells were incubated with COX-2 inhibitors at the indicated concentrations. The cellular lysates were analyzed by SDS-PAGE and immunoblotting. The amount of survivin, Bcl-xL and XIAP was quantified by densitometric analysis and was calculated in relation to the control cell after normalizing for the amount of α tubulin. The ratio was presented below the band.

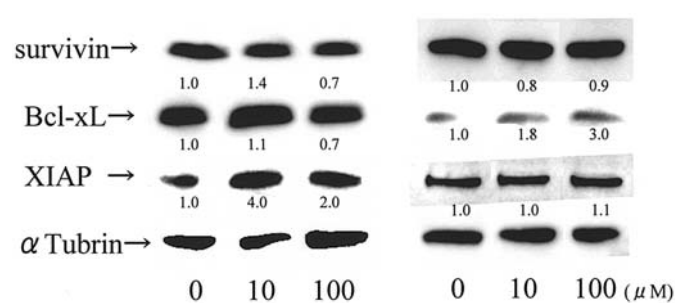


Figure 7. Expression of survivin, Bcl-xL, and XIAP in HLE cells after treatment with COX-2 inhibitors. HLE cells were incubated with COX-2 inhibitors at the indicated concentrations. The cellular lysates were analyzed by SDS-PAGE and immunoblotting. The amount of survivin, Bcl-xL and XIAP was quantified by densitometric analysis and was calculated in relation to the control cell after normalizing for the amount of α tubulin. The ratio was presented below the band.

and a variety of anti-tumor effects that might be exerted by COX-2 inhibitors have been proposed (17). For example, the anti-tumor effects of selective COX-2 inhibitors have been attributed to both anti-angiogenesis and pro-apoptosis

mechanisms (12). Indeed, COX-2 induces angiogenesis through accelerated production of both vascular endothelial growth factor (VEGF) and prostaglandins (24-26). Elevated expression of COX-2 in tumors is associated with an

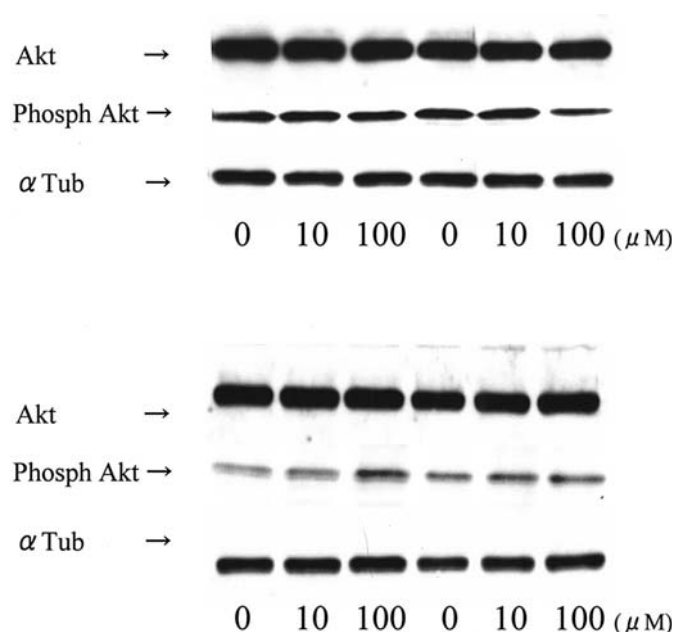


Figure 8. Expression of AKT and phospho-AKT in HCC cells. SK-Hep1 and HLE cells were incubated with COX-2 inhibitors at the indicated concentrations. The cellular lysates were analyzed by SDS-PAGE and immunoblotting.

increase in angiogenesis, invasiveness, and resistance to apoptosis. Moreover, COX inhibitors caused an increase in the detectable levels of the von Hippel Lindau protein, which participates in ubiquitination of the hypoxia-inducible factor-1 α (HIF-1 α) protein and eventually leads to down-regulation of VEGF via HIF-1 α degradation (27). In addition, COX inhibitors are known to act as agonists for peroxisome proliferator-activated receptors (PPARs) α and γ , which are members of the nuclear hormone receptor superfamily. COX inhibitors have strong anti-inflammatory and anti-angiogenic effects, extending the repertoire of potential targets of PPAR γ ligands beyond cell autonomous mechanisms of cancer. However, the molecular and biochemical pathways responsible for the apoptotic effects of COX-2 inhibitors remain poorly understood.

Previous studies have shown that although most HCC cell lines are resistant to TRAIL, co-treatment of chemotherapeutic agents and irradiation made TRAIL-resistant cells sensitive to TRAIL. We have found that subcytotoxic levels of COX-2 inhibitors sensitize HCC cells to TRAIL-induced apoptosis. These results suggest that COX-2 inhibitors modulate the apoptotic pathway to bring about an increase in cell sensitivity to TRAIL receptor-mediated signaling. In support of this idea, we found that COX-2 inhibitors caused a significant upregulation of TRAIL-R2/DR5 and, to a lesser extent, upregulated TRAIL-R1/DR4. Previous reports also suggested that, in human colon cancer cell lines, overexpression of COX-2 inhibitors reduces TRAIL-R2/DR5 mRNA and protein whereas sulindac sulfide increased both TRAIL-R1/DR4 and TRAIL-R2/DR5 mRNA (30,31). Induction of TRAIL-R1/DR4 and TRAIL-R2/DR5 mRNA following DNA damage is dependent on the presence of wild-type p53; however, in our study, SK-Hep1 and HLE, which lack a wild-type copy of p53, both exhibited TRAIL-

R1/DR4 and TRAIL-R2/DR5 up-regulation. These results provide support for the idea that up-regulation of TRAIL-R1/DR4 and TRAIL-R2/DR5 by COX-2 inhibitors is p53-independent. Moreover, activation of TRAIL-R2/DR5 has been shown to increase TRAIL-induced apoptosis both *in vitro* and *in vivo*, indicating that it is primarily TRAIL-R2/DR5 that determines the apoptotic sensitivity of TRAIL (32). Therefore, the fact that we found an increase in TRAIL-R2/DR5 expression after treatment of HCC cells with COX-2 inhibitors could explain the increase in TRAIL sensitivity.

We next looked at the inhibitors of apoptosis proteins (IAPs) in TRAIL signaling, since these proteins play an important role in determining the sensitivity of apoptosis in HCC cells. The strongest evidence for the involvement of IAP family proteins in cancer has been found for two family members, survivin and XIAP (33,34).

We found that both inhibitors were able to cause a significant down-regulation of survivin in both of the cell lines. In addition, we have shown that survivin is overexpressed in human HCC and that it is a principal inhibitor of apoptosis, presumably via its ability to inhibit caspase-3 and -7. Previous studies suggested that survivin can directly bind to caspases. From our results, we conclude that down-regulation of levels of survivin protein by COX-2 inhibitors is one cause of the increase in TRAIL-induced apoptosis that we observed for HCC cells. Interestingly, another IAP, XIAP, was also down-regulated by NS398 in SK-Hep1. Suppression of survivin by COX-2 inhibitors has also been reported in non-small cell lung cancer cells. Further study will be needed to understand how these IAP family proteins are regulated by COX-2 inhibitors (35).

Protein levels of the other caspase inhibitor, Bcl-xL, were also significantly down-regulated by treatment with the COX-2 inhibitors. Most anticancer drugs induce apoptosis by engaging the mitochondrial pathway, which is regulated by Bcl-2 family proteins. Bcl-xL is overexpressed in human HCC cells and has been shown to be a principal anti-apoptotic factor induced by cellular stresses such as staurosporine treatment, serum starvation, and p53 activation. Thus, we consider down-regulation of Bcl-xL another important cause of the augmentation of TRAIL-induced apoptosis.

Finally, we investigated the AKT pathway, since the serine/threonine kinase AKT (protein kinase B) is a regulator of cell survival and apoptosis, and its activation has been shown to protect a variety of cells against apoptosis. AKT promotes cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets, including BAD and caspase-9. Our study demonstrated that the level of phosphorylated AKT was reduced by NS398 in SK-Hep1. Recently, celecoxib was reported to induce apoptosis by blocking the activation of anti-apoptotic AKT in prostate cancer cells via an action that is independent of Bcl-2 (36). Previous studies also indicated that, of six selective COX-2 inhibitors, only celecoxib caused a decrease in the level of phospho-AKT in HT-29 and SW-480 cells (37). However, the mechanism by which celecoxib suppresses AKT phosphorylation is still unknown, although celecoxib inhibited PDK1 and PTEN phosphorylation in cholangiocarcinoma cells (38). Thus, inhibition of AKT phosphorylation may be dependent on which COX-2 inhibitor is tested and on cell-type.

In conclusion, we demonstrated that COX-2 inhibitors can induce apoptosis and augment TRAIL sensitivity by up-regulation of TRAIL receptors and down-regulation of survivin and AKT pathway signaling. Since sensitivity to apoptosis is closely related to resistance to conventional anticancer agents, radiotherapy, and photodynamic therapy, these results suggest that combining COX-2 inhibitors with a TRAIL agonist or other anti-cancer therapies may prove to be an effective new strategy for treatment of HCC.

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