Induction of Apoptosis and Inhibition of Cell Growth in Human Hepatocellular Carcinoma Cells by COX-2 Inhibitors

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ABSTRACT: The aim of the present study was to examine the effects of non-selective (indomethacin) and selective cyclooxygenase-2 (COX-2) inhibitors (NS-398, nimesulide, and CAY10404) on cell growth, cell cycle distribution, and apoptosis in three human hepatocellular carcinoma cell lines (HepG2, HuH-6, and HA22T/VGH) with different characteristics of differentiation and biological behavior. The four COX inhibitors showed a dose-dependent growth-inhibitory effect in all the cell lines. No substantial arrests in the progression of the cells through the cell cycle were observed after treatment of HuH-6 or HA22T/VGH for 48 h with the various inhibitors. On the other hand, there were significant increases in apoptosis, with the highest effect of cell kill being seen after treatment with indomethacin, especially in HuH-6. Our findings support the suggestion that selective or, perhaps more efficiently, nonselective COX-2 inhibitors may have potential therapeutic effects in hepatocellular carcinoma. Further studies must be carried out to better determine the possible mechanisms of these effects.

KEYWORDS: hepatocellular carcinoma; COX-2; NSAIDs; cell growth; apoptosis

INTRODUCTION

The incidence of human hepatocellular carcinoma (HCC) has increased considerably in recent years, making it one of the 10 most frequent types of malignant neoplasia. It has an incidence as high as 10 to 20 cases per 100,000 per year in Mediterranean countries, including Spain, Italy, and Greece. Furthermore, HCC is characterized by high mortality and poor, if any, response to present drug therapies. This underlines the necessity to develop new strategies for its prevention and treatment.

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Epidemiological studies have shown that treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the incidence and mortality related to colorectal, gastric, and esophageal cancers. These drugs are well-known inhibitors of the cyclooxygenases, the rate-limiting enzymes that catalyze prostaglandin synthesis. However, conventional NSAIDs nonselectively inhibit both the constitutive form, cyclooxygenase (COX)-1, and the inducible form, COX-2. The introduction of new agents that selectively inhibit COX-2 has contributed to clarifying the role of this isoenzyme, at least in some tumor types. In particular, it is now well established that COX-2 is involved in the development and progression of colon, skin, and breast cancer.[1–3] Furthermore, COX-2 is chronically overexpressed in many premalignant, malignant, and metastastic cancers, with a significant correlation to increased invasiveness, poorer prognosis, and reduced survival in some of these cancers. In a proof-of-principle clinical trial, treatment with a selective COX-2 inhibitor (celecoxib) reduced the number of colorectal polyps in patients with familial adenomatous polyposis (FAP).[4]

The role of COX-2 in hepatocellular carcinogenesis is less clear. Some studies have shown increased expression of COX-2 in patients with various liver diseases, suggesting its possible role in chronic liver disease and during the progression of HCC;[5–7] or have described the possible benefits of treatment with COX-2 inhibitors as indicated by their effects in human HCC cell lines.[6,8–12] However, contrasting results have also been published about the expression of COX-2[8,10,11] or the effects, in inhibition of cell growth and induction of apoptosis, of COX-2 inhibitors in HCC cells.[10–13] In this study, we examined the effects of either selective or nonselective COX-2 inhibitors in three human hepatoma cell lines (HepG2, HuH-6, and HA22T/VGH) expressing different levels of COX-2 mRNA and protein. However, the effects of these inhibitors on the *in vitro* growth and apoptosis of HuH-6 and HA22T/VGH cells have not yet been analyzed.

MATERIALS AND METHODS

Reagents and Cell Culture

NS-398, nimesulide, CAY10404, and indomethacin were purchased from Cayman Chemical (Ann Arbor, MI). All the reagents were dissolved in dimethyl sulfoxide (DMSO). HepG2, HuH-6, and HA22T/VGH human hepatoma cells were used in this study. All had a narrow range of passage number and were maintained as previously described.[14]

Extraction of Cellular RNA and Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from HCC cells using TRIzol reagent (Invitrogen, Milan, Italy). Reverse transcriptase–polymerase chain reaction (RT-PCR) was then performed using the Superscript One-step RT-PCR kit (Invitrogen, Milan, Italy). The amount of cDNA was quantified and equalized using primers to β-actin as an internal control. To amplify COX-2 or β-actin fragments, 20, 25, and 30 cycles were used to determine whether DNA amplification was linear. All PCR products were ana-

lyzed by electrophoresis on agarose gel and photographed. The sequences of primers used in the RT-PCR were as follows:

COX-2: 5′-GAGAAAACTGCTCAACACCG-3′ (sense) and
5′-GCATACTCTGTTGTGTTCCC-3′(antisense);

β-actin: 5′-TCACCCACACTGTGCCCATCTACGA-3′(sense) and
5′-CAGCGGAACCGCTCATTGCCAATGG-3′ (antisense).

Evaluation of Cell Growth by MTS Assay

To test the effects of the agents, exponentially growing cells were suspended at 5×10^4 cells/mL in complete medium containing 1% fetal bovine serum (FBS). Then 100 μL of cell suspension were distributed into each well of 96-well microtiter plates and incubated overnight. At time 0, the medium was replaced with fresh complete medium containing 1% FBS, the agents were added, and the cells were cultured for additional time periods. At the end of treatment with various concentrations of inhibitors, 15 μL of a commercial solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (Promega Corporation, Madison, WI) was added. The plates were incubated for 2 h in a humidified atmosphere at 37°C in 5% CO_2. The bioreduction of the MTS dye was assessed by measuring the absorbance of each well at 490 nm. Cytotoxicity was expressed as a percentage of the absorbance measured in the control cells. The IC_{50} value of each inhibitor was determined as the concentration at which we observed a reduction in cell viability equal to 50% of the value of the control cells. Values were expressed as means ± SD of three separate experiments, each of which was performed in triplicate.

Evaluation of Cell Cycle Distribution and Apoptosis by Flow Cytometry

After 24 h of treatment, cells were washed twice with ice-cold PBS and then resuspended at 1×10^6/mL in a hypotonic fluorochrome solution containing 50 μg/mL propidium iodide in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40. After 1 h of incubation in this solution the samples were filtered through nylon cloth (40-μm mesh), and their fluorescence was analyzed as single-parameter frequency histograms using a FACSort instrument (Becton Dickinson, Mountain View, CA). The data were analyzed with CellQuest software (Becton Dickinson), and the percentage of cells in each phase of the cell cycle was determined. Apoptosis was determined by evaluating the percentage of events accumulated in the preG_o-G_1 position. The occurrence of apoptosis was also evaluated by studying phosphatidylserine exposure on the cell surface. The cells were resuspended at 1×10^6/mL in binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 μM NaCl, and 2.5 mM $CaCl_2$), incubated with FITC-conjugated annexin V (Pharmingen, San Diego, CA), and then analyzed by flow cytometry.

RESULTS

We first characterized the HCC cell lines for their expression of COX-2. The expression of COX-2 mRNA was evaluated by semiquantitative RT-PCR, using total

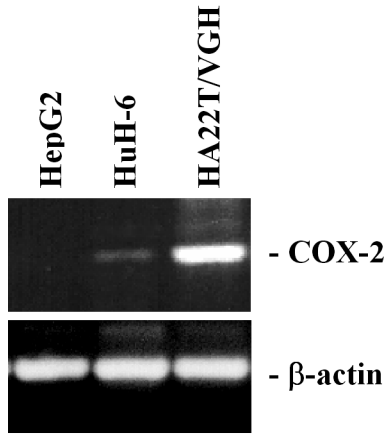

FIGURE 1. Expression of COX-2 in hepatoma cell lines. Total RNA was isolated and RT-PCR was performed as indicated in MATERIALS AND METHODS. β-actin levels are shown as an internal control. The results shown are in the linear range of PCR amplification. The data shown are representative of two independent experiments.

RNA extracted from exponentially growing cultures of HepG2, HuH-6, and HA22T7VGH cells. As shown in FIGURE 1, COX-2 mRNA was expressed strongly by HA22T/VGH cells, weakly by HuH-6 cells, and not at all by HepG2 cells. However, when we performed a second round of PCR from the HepG2 samples, we found the expected amplification product, suggesting that HepG2 cells also express COX-2 mRNA, albeit at very low levels (data not shown). Similarly, Western blot analyses revealed that in the cell lines studied, HA22T/VHG had the highest and HepG2 the lowest expression of COX-2 protein (data not shown).

To determine the effects of COX inhibitors on cell growth, we added various doses of nonselective (indomethacin) and selective (NS-398, nimesulide, and CAY10404) COX-2 inhibitors to the culture medium of the HCC cell lines. CAY10404 is a new compound from Cayman Chemical Company (Ann Arbor, MI), endowed with a high (>500,000) selectivity index (IC_{50}COX-1/IC_{50}COX-2) in favor of COX-2 inhibition. To our knowledge, the effects of this compound had never been tested on human HCC cells. The compounds showed varying degrees of cell growth inhibition in the cell lines as measured after 72 h by MTS assay (FIG. 2), with HuH-6 cells being the most sensitive (TABLE 1). The IC_{50} and IC_{70} values showed that CAY10404 was the most potent of the agents in HepG2 and HuH-6 cells. These cells were approximately twofold more sensitive to the compound than HA22T/VGH cells. With indomethacin, IC_{50} values were similar to those of the selective inhibitor CAY10404, especially in HuH-6 cells (TABLE 1).

Studies have shown that COX-2 inhibitors suppress cell cycle progression and increase the rate of apoptosis in neoplastic cells. Therefore, using flow cytometry analysis of DNA stained with propidium iodide, we studied distribution in the phases of the cell cycle and the occurrence of cell death in the two most sensitive cell lines,

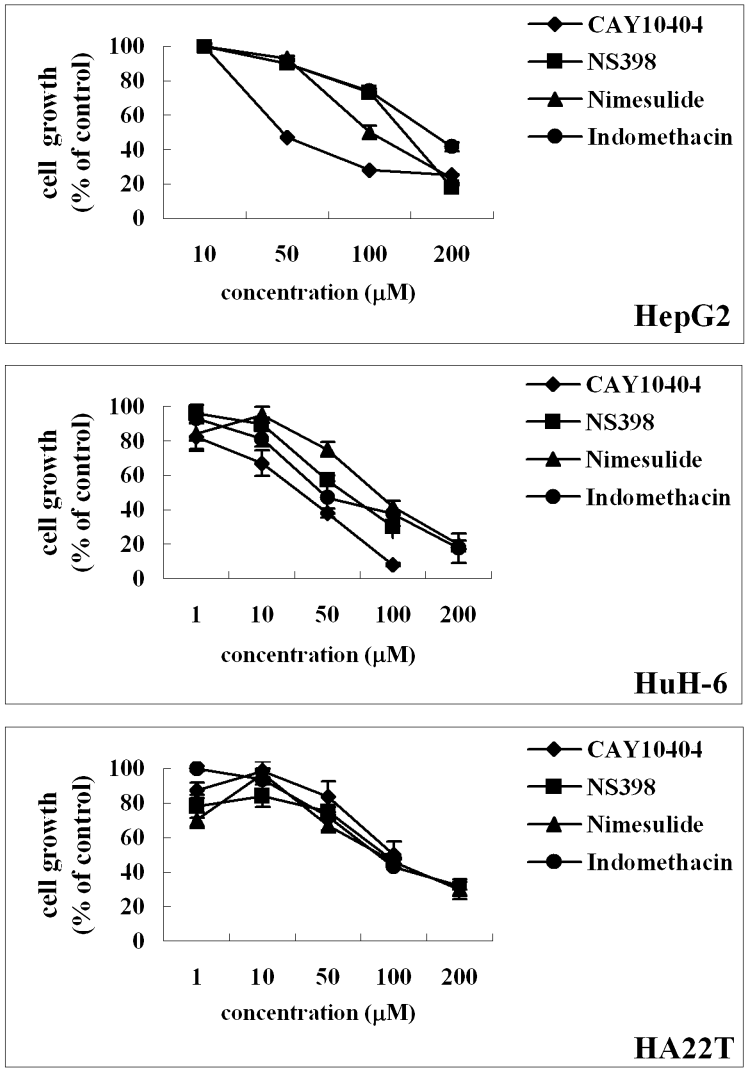

FIGURE 2. Growth inhibition induced by COX inhibitors. Cells were incubated in the presence of the indicated concentrations of the inhibitors for 72 h. Data are expressed as percentage of untreated cells. Values are means ± SD of three separate experiments.

TABLE 1. IC_{50} (μM) and IC_{70} (μM) values for the different COX inhibitors

	HepG2		HuH-6		HA22T/VGH	
	IC_{50}	IC_{70}	IC_{50}	IC_{70}	IC_{50}	IC_{70}
CAY10404	45	95	41	63	99	130
NS-398	146	180	61	100	88	200
Nimesulide	100	173	86	148	72	200
Indomethacin	170	>200	46	125	88	200

HuH-6 and HA22T/VGH, after treatment for 48 h with each of the four compounds at their IC_{50} or IC_{70} values (FIG. 3). While there were no major blocks in the progression of the cells through the cell cycle phases, we observed clear increases in the rates of apoptosis, evaluated on the basis of the percentage of events accumulated in the $preG_0$-G_1 position (FIG. 3). In both cell lines, especially in HuH-6, the highest induction of apoptosis was seen after treatment with indomethacin. Comparable results were obtained assessing apoptosis through annexin V binding (data not shown).

DISCUSSION

Hepatocellular carcinoma is one of the most common malignancies worldwide, accounting for approximately six percent of all human cancers and one million deaths annually. At present, surgical resection is the only curative therapy for HCC, but this is possible in only a minority of patients, as diagnosis is made at a late stage in most cases. Thus, the increasing incidence of HCC, along with its poor prognosis and the limited results of present therapy, emphasize the need to explore new strategies for its chemoprevention and treatment. Several lines of evidence indicate that COX-2 can be considered a pharmacological target for anticancer therapy. In fact, COX-2 affects many processes that have been implicated in different stages of carcinogenesis, including angiogenesis, inhibition of apoptosis, immune function, tumor growth, and invasiveness.

COX-2 may be a logical therapeutic target in HCC, as it can be overexpressed in patients with HCC.[5–7] There is also preliminary evidence that COX-2 inhibitors have antitumor effects in HCC cell lines.[6,8–12] However, information on the mechanisms involved in these effects is scant and sometimes contradictory. A number of studies have shown that NS-398 is able to inhibit HepG2 cell growth[6,11,12] owing to cell cycle arrest at G_1-S transition[11,12] with induction of apoptosis[6,12] or necrosis.[11] In contrast, other studies have shown that NS-398 does not affect HCC cell growth.[10,13] Also, it is still controversial whether the antitumor effects of COX-2 inhibitors in HCC are due predominantly to the inhibition of COX-2 activity.[9]

In this study, we further confirmed previous findings that COX-2 inhibitors, including the new agent CAY10404, may have potential therapeutic effects in HCC. The treatment of different HCC cell lines with selective and nonselective COX-2 inhibitors induced a dose-dependent inhibition of cell growth, which did not seem to

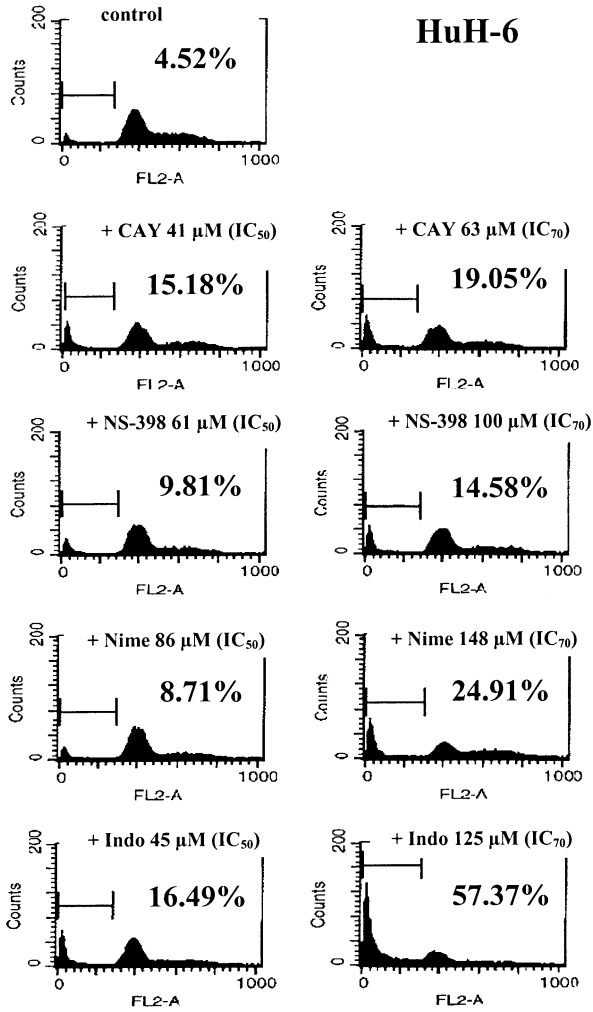

FIGURE 3. COX inhibitors induce apoptosis in hepatoma cells. Representative examples of flow cytometry analysis of apoptosis. Cells were treated with COX inhibitors at their IC_{50} or IC_{70} value for 48 h; the profiles of their propidium iodide–stained DNA are shown. Percentage of events in the $preG_o$-G_1 position is indicated in each panel.

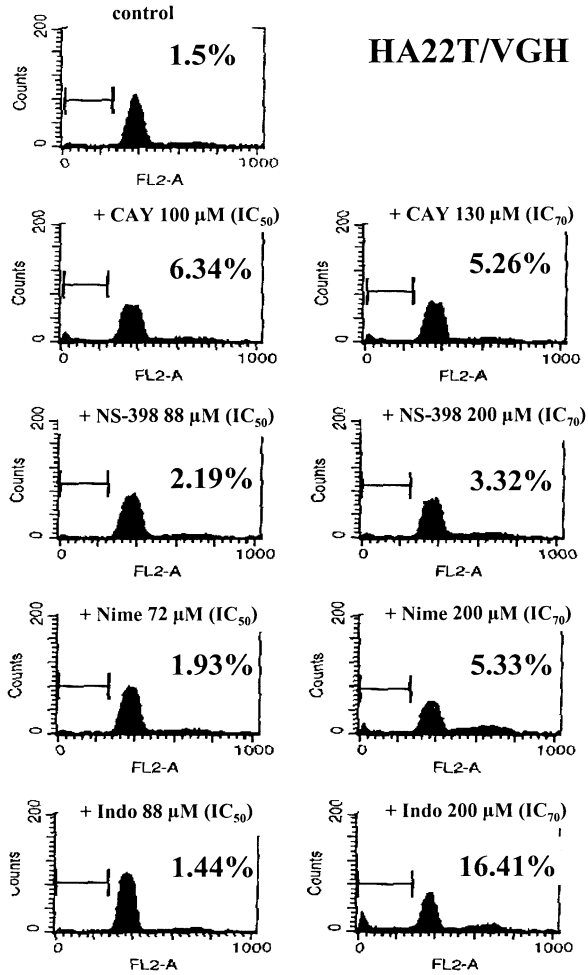

FIGURE 3 — *continued.*

be the result of evident arrests in progression through the cell cycle. In this study, cell growth inhibition was associated with significant increases in apoptosis.

Our observation that COX-2 inhibitors have cell growth–inhibitory effects on HepG2 cells, which express very low levels of COX-2, suggests that they may also act through a COX-2–independent mechanism. A similar effect has been reported for other types of cancer cells.[15–17] Clearly, further studies need to be carried out to better determine the effects of NSAIDs on HCC cells. For example, our observation that the nonselective NSAID indomethacin was able to induce more apoptosis than the selective COX-2 inhibitors in the HCC cells suggests that COX-1 also influences this process in HCC. Increasing evidence indicates that COX-1 plays an essential role in skin, intestinal, and breast tumorigenesis.[18–20] Much less attention has been paid to the possible role of the COX-1 isoform in HCC. In consideration of the ubiquitous constitutive expression of COX-1, perhaps this possibility also needs to be examined more extensively.

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