Inhibition of cyclooxygenase (COX)-2 elicits therapeutic effects in solid tumors that are coupled with the inhibition of cell proliferation and induction of apoptosis in tumor cells. Aim: This study was designed to investigate the role of COX-2 inhibitor nimesulide in cell growth and apoptosis of the cultured human hepatocellular carcinoma HepG2 cells. Methods: We performed the MTT assay, flow cytometric analysis and cell morphology study to evaluate growth inhibition and cell apoptosis upon the action of nimesulide alone or along with doxorubicin, a common agent for the treatment of human hepatocellular carcinoma. Results: Our results showed that the treatment of HepG2 cells with more than 50 μM of nimesulide suppressed COX-2 enzyme activity because of reduced PGE₂ production, and then induced growth inhibition and cell apoptosis despite no alterations of COX-2 protein expression. Importantly, the combination of 50 μM or 100 μM of nimesulide and low concentrations (5 μM to 20 μM) of doxorubicin resulted in enhanced cell growth inhibition, apoptosis induction and reduced VEGF production. Conclusion: These data suggest synergistic and/or additive effects of COX-2 inhibitors and chemotherapeutic agents, and may provide the rational for clinical studies of COX-2 inhibitors on the treatment or chemoprevention of human hepatocellular carcinoma.

Key Words: hepatocellular carcinoma, nimesulide, COX-2, doxorubicin, apoptosis, VEGF.
effective agents such as doxorubicin with complementary mechanisms remains unknown.

In this study, we have characterized the effects of COX-2 inhibition on cell growth and survival by treatment with nimesulide, a COX-2 specific inhibitor, in hepatocellular carcinoma cell line HepG2. Our results showed that the treatment with nimesulide enhanced doxorubicin-mediated cytotoxicity and reduced production of PGE₂ and VEGF. Our results that show additive inhibitory effects of COX-2 inhibitor nimesulide and doxorubicin on the growth of hepatocellular carcinoma cells may provide the rationale for clinical studies of COX-2 inhibitors on the treatment or chemoprevention of HCC.

**MATERIALS AND METHODS**

**Reagents.** Human HepG2 hepatocellular carcinoma cell line was obtained from The Research Institute on Hepatoma of Shanghai. RPMI 1640, DMEM and MEM culture medium was purchased from Gibco BRL. Fetal bovine serum (FBS) was provided by Sijiqing Biological Engineering Material (Hangzhou, China). Rabbit anti-COX-2 was from Cayman. Sheep anti-rabbit, sheep anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), nimesulide and acridine orange (AO) were purchased from Sigma Chemical Co. (St. Louis, MO). Dextrorubicin was provided by Wanle Co. (Shenzhen, China).

**Cell culture.** HepG2 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. HepG2 cells were cultured at 5% CO₂ and 37 °C in a humidified incubator (Nacpo-6100, DuPont Company, USA).

**MTT assay.** The number of viable cells was determined by MTT assay. Each cell sample was plated at a density of 10⁴ cells/well in 96-well cluster dishes for 24 h before treatment. Then the medium was changed and nimesulide and doxorubicin were added in various concentrations and analyzed at indicated time points. After addition of MTT solution (5 g/L) in each well, the cells were incubated at 37° for 4 h, and then 150 µL DMSO was added to dissolve the dark blue crystals. The absorbance was measured in an ELISA plate reader (EL301 Strip reader, BIOTEK, USA) with a test wavelength of 570 nm, and the relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. Percent cytotoxicity was calculated using the formula: percent cytotoxicity = [1 -(absorbance of experimental wells/absorbance of control wells)] × 100%.

**Cell morphology.** After treatment with drugs, cytological morphology changes were observed under the Olympus optical microscope. Cells were subcultured on coverslips in 6-well culture plates. After 24 h, the coverslips were taken out and stained with acridine orange. Cells were observed and photographs were taken under fluorescence microscope.

**Flow cytometry.** HepG2 cells were seeded in culture flasks. The culture bottles were divided into one medium alone and three drug-treated groups. Each group was in triplicate. When the cells were anchored to the plates, various drugs were added and the cells incubated at 37°, 5% CO₂, for 48 h. Then each group of cells were washed with PBS, trypsinized and then stained with propidium iodide (PI, Sigma, USA). The red fluorescence of DNA-bound PI in each group was measured at 488 nm by FACSscan flow cytometry (EPTCS XLL2MCL Beckman Coulter, UK).

**Western blot analysis.** For immunoblot analysis of COX-2, HepG2 cells were lysed in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 1% Tween 20, 2 mM EDTA, 1 mM phenylmethylsulfonil fluoride, 10 µg/mL L-protinin, and 10 µg/mL leupeptin. Debris was removed by centrifugation (Beckman GPKR machine) at 2,200 g for 20 min at 4 °C. A total of 60 µg of precipitated and denatured protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene fluoride membranes (Immun-Blot PVDF membrane, 0.2 µm; Bio-Rad). Membranes were blocked with 5% nonfat milk powder in 0.5 % Tween 20-PBS for 2 h. Primary antibodies to COX-2 were used at a dilution of 1: 1000 for overnight, at 4 °C. The blot was washed several times with 0.05% Tween 20-PBS and incubated with the appropriate goat anti-rabbit antibody for 2 h. Immunodetection was carried out using enhanced chemiluminescence reagent following the manufacturer’s instructions (Pierce Biotechnology Inc, USA).

**Measurement of prostaglandin E2 and VEGF production.** HepG2 carcinoma cells were plated at a density of 5 × 10⁴ cells /p60 Petri dish in complete DMEM medium for 12 h. HepG2 cells were treated with 25–100 µM nimesulide for 24 h. After drug treatment, culture supernatant was collected and centrifuged briefly. The amount of PGE₂ in the medium was measured using a commercial RIA kit (Wuhan Boster Co, Wuhan, China) and VEGF level determined with an enzyme-linked immunosorbent assay (ELISA) kit (Promega Corporation) following the manufacturer’s instructions.

**Statistical analysis.** Data from the population of cells treated with different conditions were analyzed using paired Student’s t-test or ANOVA test (a comparison of multiple groups), and p value of < 0.05 was considered statistically significant in the experiments.

**RESULTS**

Nimesulide inhibits the COX-2 activity in HepG2 hepatocellular carcinoma cells. First, we performed the Western blot analysis to examine whether COX-2 protein is expressed in HepG2 cells. As shown in Fig. 1, a, COX-2 protein was highly expressed in HepG2 cells. This is consistent to a recent immunohistochemistry study that shows high COX-2 protein expression in HepG2 and Huh7 cells [28]. As treatment with 25–100 µM nimesulide for 24 h did not significantly alter expression levels of COX-2 protein in HepG2 cells (Fig. 1, a), next
we tested whether treatment with nimesulide inhibits the COX-2 enzyme activity by measuring PGE\(_2\) levels of culture supernatant. The average amount of PGE\(_2\) in the medium alone controls from three independent experiments was 0.65 ng/ml. Compared with the control, the PGE\(_2\) production was reduced by about 80%, 65% and 40% 24 h after the treatment with 25 \(\mu\)M, 50 \(\mu\)M or 100 \(\mu\)M of nimesulide, respectively (Fig. 1, b), indicating the dose-dependent inhibition.

**Fig. 1.** Effects of nimesulide on the COX-2 protein expression and PGE\(_2\) production in human HepG2 cells. (a) HepG2 cells were treated with 25–100 \(\mu\)M nimesulide for 24 h as indicated. The total protein extracts were subjected to Western blot analysis using anti-COX-2 antibody. Anti-ERK-2 antibody was used as a loading control. (b) The HepG2 cells were treated with 25–100 \(\mu\)M nimesulide. After incubation for 24 h, the PGE\(_2\) level in the culture medium was measured by a PGE\(_2\) EIA kit following the manufacturer’s protocol. The means ± S.E of three independent experiments in each treatment are shown.

**Nimesulide inhibits cell growth and enhances doxorubicin-mediated apoptosis of cultured HepG2 cells.** To determine whether COX-2 inhibition by nimesulide contributes to growth inhibition of HepG2 cells we measured the number of apoptotic cells after treatment with various doses of nimesulide by MTT assay. As shown in the left panel of Fig. 2, a, the number of viable cells started to decline by treatment with a low concentration of nimesulide (25 \(\mu\)M) and continued to drop by up to 40% at 400 \(\mu\)M of nimesulide. As expected, cell apoptosis induced by doxorubicin is more evident than that by nimesulide as treatment with 80 \(\mu\)M of doxorubicin induced death of almost all cells in the culture (Fig. 2, a, right panel). The apoptosis triggered by nimesulide or doxorubicin was shown in a concentration-dependent manner.

Because nimesulide-induced cell death is through COX-2 inhibition and doxorubicin acts as cell cycle inhibitor on triggering cell apoptosis, we hypothesize that the inhibition of COX-2 by nimesulide may enhance the doxorubicin-mediated cytotoxicity. To test this possibility, we performed the MTT assay by co-treatment of HepG2 cells with doxorubicin and nimesulide. As shown in Fig. 2, b, compared to cell treated with 5 \(\mu\)M of doxorubicin alone, addition of 25, 50 or 100 \(\mu\)M nimesulide yielded a 2 to 3.5 fold increase in cell death, respectively. This synergistic effect of nimesulide persisted even at higher concentrations of doxorubicin, 10 \(\mu\)M or 20 \(\mu\)M. Since treatment with more than 40 \(\mu\)M of doxorubicin alone already induced apoptosis of most cells, an increase in doxorubicin-mediated cytotoxicity of HepG2 cells by addition of nimesulide was limited (Fig. 2, b, right two panels).

**Fig. 2.** Co-treatment of nimesulide and doxorubicin additively inhibited the growth of HepG2 cells. (a) The cells were treated with indicated 25–400 \(\mu\)M nimesulide or indicated 5–80 \(\mu\)M of doxorubicin for 24 h. The cell survival was measured by MTT assay. Results were obtained from 3 independent experiments and the bar represents S.E. Significant changes between untreated and treated samples are marked by asterisks: *\(p<0.05\) and **\(p<0.01\). (b) The cells were co-treated with 5–80 \(\mu\)M doxorubicin alone or together with 25–100 \(\mu\)M nimesulide for 24 h. Then the cell survival was measured by MTT assay. Results were obtained from 4 independent experiments and the bar represents the S.E. Significant changes between doxorubicin alone and co-treatment with nimesulide samples are marked by asterisks: *\(p<0.05\) and **\(p<0.01\).

To further confirm the cytotoxic synergy between nimesulide and doxorubicin we performed morphology study by staining cells with acridine orange. We observed the normal morphology of drug-untreated HepG2 cells: shape of tri- or multi-angles with abundant...
cytoplasm, and large oval nuclei with dispersed chromatin (Fig. 3, a, left panel). 24 h after treatment with 100 µM of nimesulide and 10 µM of doxorubicin, HepG2 cells exhibited the typical morphology of apoptotic cells, including cell shrinkage, deep-dyed pyknotic nuclei, margination of nuclear chromatin, cytoplasmic blebbing and clusters of apoptotic bodies (Fig. 3, a, right panel). Also, we measured the DNA content by propidium iodide (PI) staining, and cell apoptosis was reflected by the appearance of a cell population with subdiploid and pre-G1 phase through flow cytometric analysis. Consistent with MTT assay, addition of 100 µM of nimesulide significantly increased a portion of subdiploid and pre-G1 phase (21.7%) compared to that of doxorubicin alone group (10.6%) (Fig. 3, b, bottom two panels). By contrast, a much smaller portion of pre-G1 phase was shown in the cells treated with nimesulide alone (Fig. 3, b, right top panel).

**Fig. 3.** Co-treatment of nimesulide and doxorubicin enhanced apoptosis induction of HepG2 cells. (a) The HepG2 cells were treated with or without nimesulide 100 µM and doxorubicin 10 µM for 24 h and stained with acridine orange. The cell morphology was observed under fluorescent microscope. (b) Flow cytometry analysis of DNA content of HepG2 cells after treated with 100 µM of nimesulide and/or 20 µM of doxorubicin for 24 h. Representative DNA histograms were from 3 independent experiments. The percentage of pre-G1 phase at various culture conditions is indicated.

**Co-treatment with doxorubicin and nimesulide reduces production of VEGF in HepG2 cells.** VEGF, one of the most potent angiogenic factors, has been shown to play a pivotal role in tumor angiogenesis, including HCC. A line of evidence reveals that the elevated COX-2 expression correlates with increased VEGF level and microvascular density in human HCCs [16, 24, 25]. However, it is not known whether the inhibition of COX-2 reduces VEGF production in hepatocellular carcinoma cells. To test this possibility, we treated HepG2 cells with 25 µM or 50 µM of nimesulide and measured VEGF levels in the culture supernatants by an ELISA. As shown in Fig. 4, treatment with 50 µM of nimesulide alone led to an about 2 fold reduction in VEGF levels and this effect was largely augmented by addition of 5 µM or 10 µM of doxorubicin. VEGF production was also significantly reduced if the cells were co-treated with doxorubicin and a lower concentration (25 µM) of nimesulide, suggesting the synergistic inhibition of VEGF production in HepG2 cells by two agents.

**Fig. 4.** Co-treatment with nimesulide and doxorubicin reduced VEGF production of cultured HepG2 cells. The cells were co-treated with 5–10 µM doxorubicin and 25–50 µM nimesulide for 48 h. The VEGF levels were determined in culture supernatants by an ELISA following the manufacturer’s protocol. VEGF secretion was significantly reduced in the co-treatment group compared with the single nimesulide or doxorubicin treated group (*p < 0.05)

**DISCUSSION**

Doxorubicin is one of the most often used drugs for treatment of HCC and single use of doxorubicin yields response rate of about 20% for unresectable tumors. However, there is no convincing evidence that the use of doxorubicin improves the prognosis of patients with HCC, therefore the effect of this drug on HCC is still limited [29–32]. NSAIDs, such as aspirin, indomethacin and sulindac, may play a role in the inhibition of proliferation and induction of apoptosis in tumor cells through the inhibition of COX-2 activity [33–35]. The anti-inflammatory and anti-angiogenic effects of NSAIDs have been explored for the cancer therapy and some of these agents are currently under clinical trials [36, 37]. Nimesulide, a specific COX-2 inhibitor, can bind specifically to the large catalytic moiety of COX-2, with much less adverse effects on the gastrointestinal tract compared to the non-specific NSAIDs. A selective COX-2 inhibitor JTE-522 has been reported to enhance cytotoxicity in bladder cancer with 5-fluorouracil [38]. A combination of common antitumor drugs and COX-2 inhibitors may enhance chemotherapeutic efficacy, reduce drug dose and adverse side effects. It has been shown that nimesulide inhibits the growth of human hepatoma cell line SMCC-7721 in vitro [17]. However, studies on the combination of selective COX-2 inhibitors including nimesulide and common chemotherapy agents against human hepatocellular carcinoma are not documented. In this study, we investigated the effects of a combination of nimesulide and doxorubicin on the growth inhibition in human hepatocellular
levels of VEGF in cultured supernatants. Together with our previous report, the combination of doxorubicin and nimesulide additively increased the cytotoxicity and growth inhibition in HepG2 cells. Therefore, our results suggest that the use of COX-2 inhibitors may be beneficial when combined with doxorubicin for the treatment of patients with HCC.

Prostaglandins, including PGE₂, synthesized by COX enzymes, are reported to increase cell growth and induce proliferation in the cultured rat hepatocytes [39, 40]. In human hepatocellular carcinoma cells, treatment with prostaglandin E₅ (PGE₅) increases VEGF production and this effect is blocked by inhibition of COX-2, suggesting a link of COX-2 with VEGF signaling in hepatocarcinogenesis [16]. In support of these findings, we have observed the high expression of COX-2 protein in HepG2 cells and inhibition of COX-2 by nimesulide led to reduced production of PGE₂ and VEGF, and decreased viable cells in the culture. Therefore, the interplay between COX-2-derived prostaglandin signaling and other growth-regulatory pathways such as VEGF is expected to provide important therapeutic implications.

Apoptosis is an important physiological process that prevents the formation of tumor clone and the failures of apoptosis lead to the development of many tumors including hepatocellular carcinoma [41]. The recent reports have demonstrated that NSAIDs induce apoptosis in different tumor cells [12, 42]. It is also conceivable that apoptosis might occur in the human hepatocellular carcinoma cells in response to nimesulide. To evaluate the role of nimesulide in cell apoptosis, we cultured HepG2 cells and treated cells with various concentrations of nimesulide or together with a low concentration of doxorubicin. Flow cytometry analysis of sub-diploid peak of DNA content revealed the ability of nimesulide to trigger apoptosis in HepG2 cells at these concentrations that were sufficient to inhibit cell proliferation. In addition, the nimesulide-induced apoptosis is significantly increased by addition of a low concentration of doxorubicin. Thus, apoptosis may be one of the mechanisms for nimesulide to inhibit cell growth in HepG2 cells, especially when nimesulide is combined with doxorubicin.

In patients with hepatocellular carcinoma, hypervascularity correlated with the over-expression of VEGF and significantly associated with the tumor extension and shorter median survival time [25, 43, 44]. Prostaglandin E₅ increases the expression and the secretion of VEGF in hepatocellular carcinoma cells [16, 45]. We found that the inhibition of COX-2 by nimesulide reduced VEGF production in HepG2 cells. Interestingly, treatment of doxorubicin also inhibited VEGF secretion by HepG2 cells, and co-treatment of doxorubicin and nimesulide markedly decreased the levels of VEGF in cultured supernatants. Together, our data suggest that co-treatment with doxorubicin and nimesulide induces growth inhibition and cell death in part through the inhibition of VEGF production in HepG2 cells. However, the precise mechanism of the inhibition of VEGF production in this situation needs further investigation.

This study for the first time presents evidence that a combination of COX-2 inhibitor nimesulide and doxorubicin additively inhibits growth of the human hepatocellular carcinoma cells. This effect was mainly observed owing to increased apoptosis and reduced VEGF production. The study provides a new strategy that the combination of COX-2 inhibitors and doxorubicin may be effective in the treatment of human hepatocellular carcinoma.

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REFERENCES


КОМБИНИРОВАННОЕ ПРИМЕНЕНИЕ ИНГИБИТОРА ЦИКЛООКСИГЕНАЗЫ-2 И ДОКСОРУБИЦИНА ПРИВОДИТ К УГНЕТЕНИЮ РОСТА И АПОПТОЗУ КЛЕТОК ГЕПАТОКАРЦИНОМЫ ЧЕЛОВЕКА

Угнетение циклооксигеназы-2 (ЦОГ-2) оказывает терапевтический эффект при лечении больных с солидными опухолями и сопровождается снижением пролиферации опухолевых клеток и индукцией апоптоза. Цель: изучение роли ингибитора ЦОГ-2 — нимесулида в процессах роста и апоптоза культивированных клеток гепатокарциномы человека HepG2. Методы: для оценки апоптоза и угнетения роста клеток при применении нимесулида самостоятельно и в сочетании с доксорубицином применяли MTT-анализ, проточную цитометрию и стандартные морфологические методы. Результаты: установлено, что обработка клеток HepG2 cells нимесулидом в концентрации > 50 μM приводила к угнетению активности ЦОГ-2 за счет снижения продукции PGE₂, после чего отмечали подавление роста и апоптоз клеток при неизменном уровне экспрессии ЦОГ-2. Комбинированное применение 50 μM или 100 μM нимесулида и доксорубицина в концентрации 5–20 μM обусловило усиленное угнетение роста клеток, индукции апоптоза и снижение продукции VEGF. Выводы: полученные данные свидетельствуют о синергическом и/или аддитивном эффекте при применении ингибиторов ЦОГ-2 и химиотерапевтических препаратов. Ключевые слова: гепатокарцинома, нимесулид, ЦОГ-2, доксорубицин, апоптоз, VEGF.