Specific COX-2 inhibitor NS398 induces apoptosis in human liver cancer cell line HepG2 through BCL-2

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Abstract
Aim: To evaluate the effects of NS-398, a cyclooxygenase-2 (COX-2) inhibitor, on the proliferation and apoptosis of HepG2 cells.

Methods: The effects of NS-398 on the proliferation of HepG2 cells were evaluated by MTT. DNA fragmentation gel analysis was used to analyze the apoptotic cells. DNA ploidy and apoptotic cell percentage were calculated by flow cytometry. The expression of COX-2 and Bcl-2 mRNA was identified by competitive RT-PCR. Furthermore, expression level of Bcl-2 was detected using Western blot in HepG2 after treated with NS-398.

Results: NS-398 inhibited cell proliferation and induced apoptosis of HepG2 cells in a concentration-dependent manner. DNA ploidy analysis showed that 5 phase cells were significantly decreased with increase of NS-398 concentration. The quiescent G0/G1 phase was accumulated with decrease of Bcl-2 mRNA. Whereas NS-398 had no effect on the expression of COX-2 mRNA, and no correlations were found between COX-2 mRNA and HepG2 cell proliferation and apoptosis induced by NS-398 (r = 0.056 and r = 0.119, respectively). Bcl-2 protein level was inhibited after treated with NS-398.

Conclusion: NS-398 significantly inhibits the proliferation and induces apoptosis of HepG2 cells. Mechanisms involved may be accumulation of quiescent G0/G1 phase and decrease of Bcl-2 expression.

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Key words: Liver cancer; NS-398; Bcl-2 protein; COX-2

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INTRODUCTION
Two forms of cyclooxygenase, Cox-1 and Cox-2, which can convert arachidonic acid to prostaglandins, have been identified. Cox-1 is constitutively expressed and synthesizes cytoprotective prostaglandins mainly in the gastrointestinal tract while Cox-2 is highly inducible by the oncoproteins ras and sc and other cytokines at the sites of inflammation and cancer. Epidemiological studies also show a lower risk of cancers of the colon, breast, esophagus, and stomach following ingestion of NSAIDs. On the other hand, classic NSAIDs inhibit both Cox-2, and Cox-1, resulting in the common side-effect of gastric mucosal damage. To reduce the gastrointestinal side-effects of NSAIDs, selective Cox-2 inhibitors have been developed, and the effect of these selective inhibitors on the proliferation and apoptosis of liver cancer cells has been the subject of investigation in recent years. However, the underlying mechanism how Cox-2 inhibitor executes anti-proliferation and pro-apoptotic effect on liver cancer cells is still unclear. To address this issue, we investigated the mechanism of Cox-2 specific inhibitor, NS-398 on the proliferation and apoptosis of HepG2 cells.
resuspended with staining solution (0.1% Triton X-100, 0.2 mg/mL RNase A and propidium iodide in PBS). The samples were analysed in a flow cytometer (Couter, USA) after incubated for 30 min at room temperature in dark.

**DNA ladder** Cell apoptosis induced by NS-398 was analyzed by agarose gel-electrophoresis. Briefly, cells (1×10⁶) were lysed with 0.5 mL lysis buffer and suspended, followed by the addition of RNase A to a final concentration of 200 µg/mL, and incubated for 1 h at 37 °C. Cells were then treated with 300 µg/mL of proteinase K for 1 h at 37 °C. After addition of 4 µL loading buffer, 20 µL samples in each lane was subjected to electrophoresis on an 1.5% agarose gel-electrophoresis. Briefly, cells (1×10⁶) were lysed with 0.5 mL lysis buffer and suspended, followed by the addition of RNase A to a final concentration of 200 µg/mL, and incubated for 1 h at 37 °C. Cells were then treated with 300 µg/mL of proteinase K for 1 h at 37 °C. After addition of 4 µL loading buffer, 20 µL samples in each lane was subjected to electrophoresis on an 1.5% agarose gel-electrophoresis.

**Reverse transcription polymerase chain reaction** To study cytokine gene expression patterns, we used competitive template reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from HepG2 cells using TRIzol reagent according to the manufacturer’s recommendations. For cDNA synthesis, 4 µg total RNA was reverse transcribed with MuLV reverse transcriptase. Sequences of the primers used for RT-PCR analysis are described in Table 1. Thirty-two cycles of amplifications were performed under the following conditions: 95 °C for 2 min, at 94 °C for 45 s, at 56 °C for 45 s, at 72 °C for 45 s. The final extension step was performed by one cycle at 72 °C for 10 min. twenty-five reaction system was used, including 2 µL cDNA template, 1 µL sense primer, 1 µL anti-sense primer, 2 µL 25 mmol MgCl₂, 1 µL dNTP and 1.5 u Taq DNA polymerase. Reaction products were run by electrophoresis on an 1.5% agarose gel for 30-40 min at 100 V in 0.5×TBE buffer, and visualized with ethidium bromide and laddering was visualized under UV light.

### Table 1: Sequences of primers for amplified cDNA of Cox-2, Bcl-2 and GAPDH

| Gene   | Primer   | Sequence                             | Length of product |
|--------|----------|--------------------------------------|-------------------|
| Cox-2  | Sense    | 5'-TGAAACCCACTCCAAACACAG-3'          | 232 bp            |
|        | Anti-sense| 5'-TCATCCAGCCAGGTCAG-3'              |                   |
| Bcl-2  | Sense    | 5'-GACTTCGCCGAGATGTCCAG-3'           | 225 bp            |
|        | Anti-sense| 5'-TCATCAGGCACAGGAGGAAG-3'           |                   |
| GAPDH  | Sense    | 5'-ATGGCACCGTCAAGGCTGAG-3'           |                   |
|        | Anti-sense| 5'-CAGGTGCCGGTTCAGGTACT-3'           |                   |

**Antibodies and Western blot** Total proteins were extracted using Western blot lysis buffer containing 5 mMol/L EDTA, 150 mMol/L NaCl, 50 mMol/L Tris HCI, 1% Triton X-100 and 0.1% SDS. The protease inhibitor cocktail prepared from protease inhibitor cocktail tablets (Roche) was added and used for no more than 2 wk. Protein extracts (20 µg) were run on 10% SDS-PAGE gels and transferred to PVDF membrane (Amersham Biosciences, USA). Following this, the membrane was blocked with 5% non-fat dry milk in TBS-T buffer for 1h and incubated with appropriate dilution ratio of first antibody overnight at 4 °C. The membranes were incubated with secondary antibody for 1 h at room temperature and detected with ECL reagent (Santa Cruz, USA).

### Statistical analysis

All data were expressed as mean±SD and analyzed by one-way of variance (ANOVA) using SPSS software (version 11.0 for Windows). Pearson correlation analysis was used between parameters. P<0.05 was considered statistically significant.

### RESULTS

**Inhibition of HepG2 cell proliferation by NS-398**

To determine the effect of the selective Cox-2 inhibitor NS-398 on HepG2 cell apoptosis, we measured the cell viability with the MTT assay for 72 h at different concentrations. As shown in Figure 1, the cell proliferation was inhibited significantly in a dose-dependent manner at the final concentrations of 50 µmol/L, 100 µmol/L and 200 µmol/L (P<0.05 vs control).

### G0/G1 arrest and apoptosis of HepG2 cells induced by NS-398

To assess the effect of NS-398 on inducing HepG2 cell apoptosis, we used DNA content test by flow cytometry. HepG2 cells were treated with NS-398 at indicated concentrations and harvested after 72 h. Then the cells were stained with propidium iodide (PI) and analyzed by flow cytometry. Figure 2A shows that NS-398, at concentrations of 100 µmol/L and 200 µmol/L, significantly increased the number of cells in G0/G1 by 67.5% and 73.1% respectively in comparison to control cells in which 61.2% of cells were in G0/G1. A reciprocal reduction in the number of cells in S phase was also observed. We detected the further effect of NS-398 on induction of HepG2 cell apoptosis. The nucleosome-sized DNA fragments, typical signs of programmed cell death, are shown in Figure 2B.

**Figure 1** Inhibition of HepG2 cell proliferation by NS-398. (mean±SD, n = 3)

**Figure 2** G0/G1 arrest and apoptosis of HepG2 cells induced by NS-398. A: G0/G1 arrest in HepG2 cells after treated with NS-398 for 72 h; B: Typical signs of programmed cell death after treated with NS-398.
Inhibition of Bcl-2 expression

Cox-2 and Bcl-2 mRNA expressions by NS-398 were confirmed by semi-quantitative RT-PCR analysis. The cDNA was prepared from HepG2 cells after treated with NS-398 at different concentrations for 72 h. Bcl-2 mRNA levels were decreased in a dose-dependent manner after treated with NS-398 while no significant change was found in Cox-2 mRNA (Figure 3A). Equal loading was confirmed by the housekeeping gene GAPDH.

Specific antibodies were used to detect Bcl-2 protein by Western blot. As shown in Figure 3B, the Bcl-2 protein levels decreased after treated with NS-398 in a dose-dependent manner.

DISCUSSION

Recently, accumulated evidence suggests that many cancers are associated with abnormal expression of Cox-2[11-13]. Several reports have shown that over-expression of Cox-2 is found in a variety of carcinomas including liver cancer[14], colon cancer[15,16], breast cancer[17,18], pancreatic cancer[19], lung cancer[20] and uterine carcinoma[21, etc. Cox-2 can be induced by a variety of proteins, including cytokines, growth factors, and tumor promoters[22,23]. It is thought that over-expression of Cox-2 can increase cell survival by inhibition of apoptosis and stimulation of angiogenesis, and enhancement of cellular adhesion to matrix proteins[24,25]. Although NSAIDs have been used in clinical cancer prevention and can induce tumor cells to undergo apoptosis[26], long-term use is limited in clinic because of its no-specific and high frequency of side-effects[27]. Therefore, selective Cox-2 inhibitors have become a focus of attention as potential chemopreventive and chemotherapeutic agents.

NS-398, one of the selective Cox-2 inhibitors, has been shown to inhibit proliferation and induce apoptosis in carcinomas including colon[28], breast, lung[29] and liver cancers[30]. But the detail mechanism is unclear. In this study, we used the HepG2 cell line to explore the mechanism of liver cancer apoptosis after treatment with NS-398.

Our data show that selective Cox-2 inhibitor NS-398 can inhibit proliferation and induce apoptosis of HepG2 liver cancer cells in a dose-dependent manner. We also found that the dose to induce apoptosis was much higher than that to inhibit expression of Cox-2, indicating that COX-2-independent actions of NS398 may exist[31]. Although most previous studies have shown that induction of apoptosis by NS398 is associated with Cox-2 expression, Cox-2 independent effects of NS398 may exist[32]. Several other studies, however, have shown that there is no correlation between COX-2 expression and apoptosis induced by NSAIDs, including NS398[33]. We compared the Cox-2 expression between the cells treated and untreated with NS-398. The results showed that expression Cox-2 was not associated with apoptosis HepG2 cells.

The Bel-2 gene has been implicated as a major player in apoptosis pathway[34]. Aspirin-induced apoptosis is associated with the down-regulation of Bel-2 expression in HEC cells[35]. We also compared the level of anti-apoptotic gene Bel-2 after treated with NS-398 and found that NS-398 could down-regulate the expression of Bel-2, suggesting that down-regulation of Bel-2 may be required for HepG2 cell apoptosis induced by NS-398, thus increasing mitochondrial permeability and cytochrome C release, which will initiate the progress of apoptosis[36].

In conclusion, selective Cox-2 inhibitor, NS-398, can suppress the growth of HepG2 cells by reducing proliferation and induction of apoptosis. Cox-2-specific inhibitor offers a potential candidate as an effective chemotherapeutic and chemopreventive strategy against human cancer.

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