Combining sorafenib with celecoxib synergistically inhibits tumor growth of non-small cell lung cancer cells in vitro and in vivo

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Abstract. Although inhibition of cyclooxygenase-2 (COX-2) or the vascular endothelial growth factor receptor (VEGFR) has been shown to be a promising antitumor strategy in non-small cell lung cancer (NSCLC), the therapeutic efficacy is limited due to inherent tumor resistance. In the present study, we selected sorafenib (SOR), a VEGFR inhibitor, in combination with celecoxib (CXB), a COX-2 inhibitor, for suppressing tumor growth and simultaneously for reducing doses of both drugs for the treatment of NSCLC. The effects of SOR combined with CXB were examined in A549 cells (an NSCLC cell line). Assays of proliferation, apoptosis, cell cycle distribution and receptor signaling were performed. We found that treatment with the combination of low concentrations of SOR and CXB significantly suppressed the proliferation of A549 tumor cells in vitro and suppressed tumor growth in vivo when compared to the actions of either agent alone. The results also showed that the combination of SOR and CXB significantly increased the induction of apoptosis and decreased the expression of inhibitor of apoptosis genes, survivin and Bcl-2 (P<0.01). Furthermore, the combination treatment significantly suppressed constitutive phosphorylation of MEK and ERK, which may contribute to the inhibition of tumor growth. Taken together, our findings revealed that this additive combination of SOR and CXB is a potential drug candidate for the treatment of NSCLC.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers (2). Although chemotherapy and radiotherapy have been widely used in the treatment of advanced NSCLC, the outcome remains unsatisfactory, with low long-term survival rates. Thus, a more effective and safer therapy for lung cancer is required (3). As increased understanding of key cellular pathways involved in tumor growth, progression and cell death has been achieved, molecular-targeted therapies have been exploited.

A large number of studies have shown that vascular endothelial growth factor (VEGF), a receptor tyrosine kinase, plays an important role in tumorigenesis, and blocking the VEGF signaling pathway can reduce tumor-associated angiogenesis and blood vessel-dependent metastasis (4,5). Therefore, a number of anti-angiogenic drugs blocking the VEGF signaling pathway (ligand or the receptors) have been developed and are currently in use in NSCLC therapy. Sorafenib (SOR), one of the small-molecule inhibitors of receptor tyrosine kinase inhibitors (RTKIs) targeting the VEGF receptor family, has been shown to have significant antitumor effects in NSCLC (5). Although it has been shown that blocking the VEGF signaling pathway inhibits angiogenesis in NSCLC, the efficacy of these drugs is often limited by unfavorable pharmacokinetics, low tumor accumulation and other adverse effects (3). Studies have shown that anti-VEGF monotherapy does not increase survival in cancer patients, compared to standard chemotherapy, yet the combination of these drugs with chemotherapy or other drugs may be an effective strategy to increase the therapeutic effect in NSCLC (6,7).

Cyclooxygenase-2 (COX-2) signaling is involved in multiple processes of tumor progression, including proliferation, survival, angiogenesis and invasion (8). Overexpression of COX-2 has been found in tissues and cell lines of NSCLC, and the COX-2 inhibitor displays inhibitory effects in advanced NSCLC patients, although its therapeutic effect is not more beneficial than chemotherapy (6). Several combined EGFR and COX-2 inhibition trials have been completed and have demonstrated promising results for head and neck squamous cell carcinoma (HNSCC) (9). Celecoxib (CXB) is a COX-2-selective non-steroidal anti-inflammatory drug (NSAID), which was found to exhibit therapeutic effects in various types of cancer. At present, CXB is widely being tested in clinical trials for its therapeutic activity against various types of cancers as a single agent and also in combination with other agents (10,11). Recently, it has been shown that the combination of SOR and a COX-2 inhibitor provides synergistic anti-proliferative and pro-apoptotic effects in human liver cancer cells (12,13). In this context, in the present study, we selected CXB as a COX-2 inhibitor in combination with SOR.
for suppressing VEGFR and COX-2 expression and simultaneously reducing the doses of both drugs for treating NSCLC.

The objective of the present study was to evaluate the feasibility of CXB in combination with SOR for inhibiting NSCLC cell growth, proliferation and angiogenesis and to reveal the underlying molecular mechanisms involved in SOR-induced apoptosis. We also determined whether treatment of CXB, used as an adjuvant agent, could allow the reduction of the dosage of SOR.

Materials and methods

Reagents. Celecoxib (CBX), one type of COX-2 inhibitor, was purchased from Pfizer Inc. (New York, NY, USA). Sorafenib (SOR) was purchased from Bayer Pharma AG (Wuppertal, Germany), and both drugs were dissolved in dimethyl sulfoxide (DMSO). For western blot analysis, the following antibodies were used: mouse monoclonal anti-B-actin (Sigma Aldrich, St. Louis, MO, USA), mouse monoclonal anti-Bcl2, mouse monoclonal anti-survivin, mouse monoclonal anti-MEK, mouse monoclonal anti-phosphorylated (p-)MEK, mouse monoclonal anti-Erk, mouse monoclonal anti-p-Erk and horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Nonidet P-40 lysis buffer, chemiluminescent peroxidase substrate, propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Aldrich; stock solutions of PI, DAPI and MTT were prepared by dissolving 1 mg of each compound in 1 ml of phosphate-buffered saline (PBS). The solution was protected from light, stored at 4°C and used within 1 month.

Cell culture. The A549 human lung adenocarcinoma cells obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) in a 5% CO2 incubator and passaged with 0.25% trypsin and 0.03% ethylenediamine tetraacetic acid (EDTA) solution.

Cell viability assay. A549 cells grown in monolayers were harvested and dispensed in 96-well culture plates in 100 µl of RPMI-1640 at a concentration of 5x10^4 cells/well. After 24 h, differential drug concentrations of SOR (0-20 µM), CBX (0-40 µM) or both (0-10 µM SOX plus 20 µM CBX) were added to the cells. Then, the cells were incubated for another 4 h. At the end of the treatment, 200 µl of DMSO was added to each well after removing the supernatant. Then, cell viability was obtained by measuring the absorbance at a wavelength of 490 nm by Thermo Multiskan MK3 microplate reader.

Measurement of prostaglandin E2 (PGE2) production. PGE2 synthesis was determined by competitive enzyme-linked immunosorbent assay (ELISA) as previously described (14). In brief, A549 cells were treated with their respective half maximal inhibitory concentration (IC50) values of SOR, CBX or both for 48 h, and then these culture media were centrifuged to remove cell debris. Cell-free culture media were collected at the indicated times, and PGE2 levels were determined by competitive ELISA as described by the kit manufacturer (Cayman Chemical, Ann Arbor, MI, USA) using an ELISA reader (μQuant; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Tumor xenograft assay. All animal experiments were performed in accordance with the institutional guidelines, following a protocol approved by the Ethics Committees of the Disease Model Research Center, The First Hospital of Jilin University. Female BALB mice, 6-7 weeks of age, were maintained under specific pathogen-free (SPF) conditions and provided with food and water ad libitum. All the animals were fed with a normal pellet diet one week prior to the experiment.

Exponentially growing A549 cells were harvested, and a tumorigenic dose of 2.5x10^6 cells was injected intraperitoneally into the 6- to 7-week old female BALB mice. Tumors were allowed to grow in the mice for 10 days, after which the animals were randomly assigned into one of four treatment groups (10 mice per group). The control group received 1% polysorbate resuspended in deionized water. The other three groups were treated with CBX (4.56 mg/kg body weight), SOX (80 mg/kg body weight) or CBX plus SOX (2.5 and 40 mg/kg body weight, respectively) intraperitoneally on alternative days for 3 weeks. The doses were selected based on previous experiments (15,16). Tumor weights were measured after the mice were sacrificed, and tumor volumes were measured before the treatment injections were administered and on day 7, 14 and 21 of the treatment. On day 22, the animals were
euthanized using chloroform, and their spleen tissues were collected and cultured for a splenocyte surveillance study. Furthermore, A549 cells were collected from the site of the treatment injection for in vivo and ex vivo cell cycle phase distribution studies.

**Assay of splenocyte proliferation.** Spleens from the treated mice were collected, and single-cell spleen suspensions were pooled in serum-free RPMI-1640 by filtering the suspension through a sieve mesh with the aid of a glass homogenizer to exert gentle pressure on the spleen fragments. Samples were washed twice in PBS 0.1% (w/v) and bovine serum albumin (BSA). After centrifugation at 200 x g for 10 min, the cells were placed into 96-well flat-bottom microplates in triplicate at 3x10^5 cells/well in RPMI-1640 supplemented with 10% FBS. The cells were then incubated in a total volume of 100 µl/well. Serum-free RPMI-1640 was used as a control. After 24 h, cell proliferation was measured by the MTT assay.

**Western blot analysis.** A549 cells were treated with their respective IC_{50} values of SOR, CXB or their combination for 48 h. The cells were then homogenized in a lysis buffer (Tris-HCl 50 mmol/l, EDTA 5 mmol/l, NaCl 150 mmol/l, sodium deoxycholate 1%, Na_{2}VO_{4} 500 µmol/l, Triton X-100 0.5%, AEBSF 10 µmol/l, NaF 10 mmol/l) on ice. The homogenates were then centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatants were collected for protein concentration determination using the Bradford reagent (Sigma). Cell extracts (50 µg of protein) were separated on a sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and transferred to nitrocellulose membranes, which were blocked in 3% BSA for 2 h. After blocking, the membranes were incubated with primary antibodies overnight at 4°C for 2 h, and then with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Proteins were visualized by exposing the chemiluminescence substrate (Sigma) to X-Omat AR autoradiography film (Eastman Kodak, Rochester, NY, USA). Blots were stripped and reprobed with antibody for 2 h at room temperature. Beta-actin was used as a loading control.

**Statistical analysis.** Statistical analyses were undertaken using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA) and the SPSS® Statistical Package, version 19.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Data are expressed as the mean ± SD. The statistical significance was determined using one-way analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant result.

**Results**

**Effects of SOR and CXB alone or in combination on NSCLC cell growth.** To evaluate the effect of SOR, CXB and their combination on the cell viability of NSCLC cells in vitro, A549 cells were treated with increasing concentrations of CXB (0-40 µM) or SOR (0-20 µM). SOR inhibited cell proliferation of A549 cells dose-dependently with an IC_{50} of 4.4±0.18 µM. CXB also reduced cell viability of A549 cells in a dose-dependent manner with an IC_{50} of 25.5±0.79 µM. Combination treatment (0-10 µM in the presence of 20 µM CXB) reduced the cell viability of A549 cells in a dose-dependent manner with an IC_{50} of 2.8±0.79 µM. Based on these results, we chose the respective IC_{50} values of the drugs for further treatments throughout the study.

We next examined whether the combination of relatively low concentrations of SOR and CXB could additively or synergistically inhibit HCC cell growth in vitro using the respective IC_{50} values of SOR and CXB. As shown in Fig. 1, the inhibitory rate of the combination treatment was higher than the rates after single drug treatments (P<0.01). There was no significance different between the SOR-treated group and CXB-treated group (P>0.05).

**Effects of SOR and CXB alone or in combination on NSCLC cell apoptosis and cell cycle.** To investigate whether SOR and CXB alone or their combination could induce apoptosis, we analyzed the apoptosis after treatment with SOR and/or CXB. Treatment with the combination of these drugs led to a marked increase in apoptotic cells when compared to the extent of apoptosis following treatment with the single drugs (P<0.05) (Fig. 2A). In addition, there was no significance different between the SOR-treated group and CXB-treated group in regards to induction of NSCLC cell apoptosis.

The effects of SOR and CXB on the cell cycle distribution of A549 cells were then analyzed by flow cytometry. A549 cells treated with SOR or CXB demonstrated an increased percentage of apoptotic cells (cell cycle arrest at the G0/G1 phase) compared with the untreated cells (Fig. 2B). The low-dose combination resulted in an even greater percentage of apoptotic cells when compared with the percentage of apoptosis following treatment with higher doses of either drug alone (P<0.01). These data are consistent with the results from the acridine orange (AO) staining assay. In conclusion, these results indicate an additive mechanism of SOR and CXB in inducing cell death through apoptosis.

In order to explore the possible mechanism of the pro-apoptotic effect of the combination treatment with SOR and CXB, expression patterns of survivin and Bcl-2 were determined by western blot analysis. The results (Fig. 2C and D)
showed that treatment with the combination of CXB and SOR significantly decreased the expression of inhibitor of apoptosis genes, survivin and Bcl-2, in A549 cells, when compared to the expression levels in cells treated with CXB or SOR alone (P<0.01).

**Effects of SOR and CXB alone or in combination on the PGE2 production in NSCLC cells.** To examine the effect of SOR and CXB on PGE2 production in A549 cell, ELISA was performed. As shown in Fig. 3, CXB inhibited PGE2 production; however, CXB in combination with SOR or SOR alone did not inhibit PGE2 production, suggesting that the synergy between CXB and SOR did not extend to COX-2-dependent PGE2 production in NSCLC cells.

**Effects of SOR and CXB on the RAF/MEK/ERK signaling pathway in A549 cells.** The RAF/MEK/ERK pathway is downstream of Ras activation, and tyrosine phosphorylation of these proteins is essential for cancer cell proliferation (17). Therefore, we evaluated the effect of SOR and CXB alone and in combination on the phosphorylation of these proteins by western blotting (Fig. 4). We compared the phosphorylation of these proteins in cells treated with the respective IC_{50} values of SOR and CXB alone and in combination for 2 h. It was
found that SOR and CXB alone or in combination inhibited the tyrosine phosphorylation of MEK and ERK (Fig. 4). In addition, following treatment with the combination of SOR and CXB, a greater reduction in phospho-MEK and ERK was caused than what was observed by either agent alone.

**SOR plus CXB causes significant inhibition of tumor growth.** We assessed the *in vivo* therapeutic efficacy of SOR and CXB in female BALB mice bearing A549 cell tumors. Mice were sacrificed and tumor tissue was obtained 21 days after treatment. The tumor weight of each animal was measured. It was found that the tumor weight in the mice treated with the combination of SOR and CXB was lower than the tumor weight of the untreated group and single drug-treated groups (P<0.01) (Fig. 5A). In addition, we found that the tumor growth rate after treatment with the combination of CBX and SOR was significantly reduced when compared with the single drug-treated groups and the untreated group (P<0.01) (Fig. 5B). These results indicate that the combination treatment of SOR and CXB suppressed the tumorigenicity of A549 cell-derived tumors in mice.

**Combination of SOR and CXB inhibits splenocyte proliferation.** To assess the efficacy of SOR and CXB in modulating splenocyte proliferation, spleen cells of the treated A549 mice were isolated and cultured in RPMI-1640 containing 10% fetal bovine serum for 24 h and subjected to *in vitro* proliferation assays using MTT assay. As shown in Fig. 6, the inhibitory growth rate of cell proliferation following treatment with the combination of SOR and CXB was higher than this rate in the single drug-treated groups, which demonstrated that the combination treatment inhibited A549 cell proliferation.

**Discussion**

Recently, studies have shown that VEGF plays an important role in angiogenesis of NSCLC, and blocking the VEGF signaling pathway is an effective means to inhibit angiogenesis in animal models of NSCLC and in NSCLC patients. Although targeting the VEGF signaling pathway has been shown to be a promising antitumor strategy, treatment effectiveness remains unsatisfactory (18). COX-2 inhibitors have been demonstrated to have an antitumor effect in NSCLC cells and patients (6). Recently, studies have shown that a combination with different drugs to treat tumor patients may increase the efficiency of the antitumor response. In the present study, we hypothesized that treatment with the combination of CBX and SOR could enhance the inhibitory effect on NSCLC cells. The results showed that the combination of CBX and SOR significantly increased the growth inhibition rate and the apoptosis rate of A549 cells.

Previous studies have shown that SOR alone significantly inhibits the growth of NSCLC cells, and its combinations with other drugs, such as gemcitabine and erlotinib, could increase the inhibitory effect on NSCLC cells or models (19,20). COX-2 inhibitors, CBX or N398, have also been shown to promote the apoptosis of NSCLC and other cancer cells (21,22). Since COX-2 inhibition promotes interferon (IFN)-γ-dependent enhancement of antitumor responses, the role of COX-2 inhibitors in advanced NSCLC has been evaluated in numerous studies (6,21). However, COX-2 inhibition monotherapy did not enhance the treatment effectiveness, while its combination with chemotherapy or other target drugs exhibited a synergistic antitumor effect (6). To the best of our knowledge, the results of the present study first demonstrated that CBX and SOR provide synergistic anti-proliferative and pro-apoptotic effects in NSCLC cells. Recently, the synergistic anti-proliferative and pro-apoptotic effects of CBX and SOR were confirmed in human liver cancer cells (10). Although the
results are encouraging, the antitumor effects must be further investigated in NSCLC models and patients.

In order to explore the possible mechanism of the synergistic effects of CBX and SOR against NSCLC cells, the expression of survivin and Bcl-2 was assessed using western blot analysis. The results showed that the combination of CBX and SOR significantly decreased the expression of these critical inhibitors of apoptosis proteins. It is well known that multiple genetic pathways are involved in the regulation of cell apoptosis. Survivin and Bcl-2 are critical inhibitors of apoptosis proteins. Survivin inhibits cell apoptosis by acting on caspase directly or indirectly. Previous studies have demonstrated the expression of COX-2 and its prognostic significance in NSCLC (23). The COX-2 inhibitor alone was found to inhibit the expression of survivin and Bcl-2 in NSCLC cells (22). Similarly, our results showed that CBX or SOR alone decreased the expression of these two proteins, and the combination of CBX and SOR further decreased the protein expression. This result is consistent with the results of cell proliferation and apoptosis. In addition, the synergistic effect of CBX and SOR may be due to the enhancement of inhibitory action on the VEGF signaling pathway by the COX-2 inhibitor. It has been shown that treatment with a COX-2 inhibitor leads to restricted angiogenesis and decreased production of VEGF (24). RNA-mediated knockdown of COX-2 was found to inhibit the growth and expression of VEGF in human osteosarcoma (25).

Accumulating evidence shows that the role of CBX when administered in combination with other drugs in cancer therapy is modulatory rather than therapeutic, and the efficacy of this approach has been reported for various types of cancers (13, 26–28). Our results indicated that PGE2 was not involved in the combination effect of CBX with SOR in the present study, which further confirmed this concept.

MAPKs (mitogen-activated protein kinases) including extracellular signal-regulating kinase (ERK), p38 MAPK and c-Jun N-terminal protein kinase (JNK) play important roles in cell proliferation, apoptosis, and many other nuclear events (29). Accumulating evidence indicates that alterations of the activities of MAPKs are involved in the effects of antitumor agents in various cancer cell lines (17, 30–32). Therefore, targeting of the MAPK pathways is a promising strategy for NSCLC treatment. In the present study, to the best of our knowledge, we first examined the intracellular signaling pathway in A549 cells following treatment with the combination of SOR and CBX. Our results showed that SOR combined with CBX inhibited the Ras/Raf/MAPK pathway in A549 cells, which is in agreement with previous studies that SOR inhibits cancer cell growth through phosphorylation of p-MEK (12, 13, 33, 34). Thus, the combination of SOR with CBX may target the Ras/Raf/MAPK pathway and this targeted approach may underlie the synergistic effects revealed here.

In conclusion, the present study showed that SOR in combination with CBX enhances the anti-proliferative and pro-apoptotic effects on NSCLC cells via actions on the anti-apoptotic RAF signaling pathways. Celecoxib strengthens the anti-proliferative action of sorafenib, promoting NSCLC cell apoptosis and allowing for the use of lower doses of sorafenib than those currently used. Therefore, it is worthwhile to consider this combination treatment for NSCLC and warrants further evaluation in clinical trials.

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References