Original Article

S-1 and celecoxib synergistically suppress pancreatic cancer growth by promoting apoptosis in vivo and in vitro

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Abstract: Objective: Selective cyclooxygenase-2 (COX-2) inhibitor celecoxib has been found to suppress the development of hepatocellular carcinoma and pancreatic cancer. S-1 is a new generation of oral fluorouracil anti-cancer drug. The aim of this study is to investigate the synergistic role of S-1 and celecoxib in pancreatic cancer growth. Methods: The anticancer activities of S-1, celecoxib and their combination were investigated in vitro with human pancreatic cancer PANC-1 cells by analyzing cell viability with MTT assays, apoptosis and cell cycle pattern via flow cytometry and fluorescence microscopy in addition to migration and cell adhesion assays. Bcl-2 and survivin, Bax, caspase-8, caspase-3 and cleaved PARP activities were determined by quantitative real-Time PCR (qRT-PCR), western blotting and immunohistochemistry assays. In vivo measurements comprised a mouse PANC-1 xenograft assay. Results: S-1 combined with celecoxib synergistically inhibited the viability of PANC-1 cells in a dose-dependent manner. Combined S-1 and celecoxib application inhibited cell cycle progression, induced apoptosis, down-regulated Bcl-2 and survivin, up-regulated Bax, caspase-8, caspase-3 and cleaved PARP activities as well as reduced cell-matrix adhesion and migration to higher levels as their mono-applications. Murine subcutaneous PANC-1 xenograft growth rates were reduced by 32.61% with S-1, 39.53% with celecoxib and 73.13% with S-1 and celecoxib combined applications. Conclusions: S-1 and celecoxib synergistically suppressed PANC-1 viability by up-regulating the pro-apoptotic protein Bax and down-regulating anti-apoptotic proteins Bcl-2 and survivin, thus triggering the intrinsic caspase signaling. The results may provide a theoretical basis for the clinical use of S-1 and celecoxib in combination for pancreatic cancer treatment.

Keywords: Pancreatic cancer, S-1, celecoxib, apoptosis, survivin

Introduction

Pancreatic cancer is one of the most aggressive gastrointestinal malignancies and the fourth leading cause of carcinoma-related mortality. There were 45,220 estimated new cases in 2013, and about 38,460 patients were expected to die of this disease in America [1]. The incidence and mortality of pancreatic cancer keeps increasing in China, being ranked as the seventh leading cause of cancer-related death [2]. Despite decades of effort, pancreatic cancer still keeps the status of poor prognosis, with the incidence almost the same as the mortality [3], < 6 month median survival and only a 4-5%, 5-year survival rate [4]. Although surgery offers the only chance of cure, most pancreatic patients miss the opportunity due to locally unresectable or distant metastasis, which arise due to delayed clinical diagnosis. Only 15% of pancreatic cancer patients are diagnosed at the stage that resection can be performed [5]. Even worse, in the majority of patients who underwent surgical treatment the disease will
Clinical benefits and actions of S-1 in combination with celecoxib

still recur locally or distantly after a short period of time [1, 6]. Therefore, systemic chemotherapy and/or radiotherapy have become the most important treatments for advanced pancreatic cancer patients.

However, pancreatic cancer is obviously resistant to chemo- or radiotherapy, although as the standard first-line chemotherapeutic drug for unresectable or metastatic pancreatic cancer, gemcitabine has showed some promise. Some studies have suggested that anti-tumor effect of gemcitabine monotherapy had a higher efficacy than 5-fluorouracil (FU) monotherapy, with a significant improvement in the median survival time [7].

Recently, several studies have revealed that combinations of chemotherapeutic agents targeting variant hallmarks of pancreatic cancer improved the treatment efficacy [8-10].

S-1 (S-1) is a novel oral fluorouracil anti-cancer drug, which consists of tegafur (FT, a prodrug of 5-FU), gimeracil and oteracil [11]. In 2005, S-1 was used as systemic chemotherapy against unresectable locally advanced or metastatic pancreatic cancer in Japan. A number of clinical studies confirmed that S-1 was better than other similar drugs for the treatment of pancreatic cancer. Compared with gemcitabine, oral administration of S-1 was more convenient than conventional intravenous administration; S-1 monotherapy as adjuvant chemotherapy was found to have a better objective response rate, longer median life span and lower adverse reactions [12-14].

Proinflammatory cyclooxygenase-2 (COX-2) is an established factor linking inflammation with cancer in various organs including the pancreas. Studies have suggested that COX-2 is upregulated in pancreatic cancer tissues, with a low expression or no detectable expression in the adjacent normal tissues. High expression of COX-2 was closely related with pancreatic cancer cell proliferation, angiogenesis and anti-apoptosis, and also the occurrence and development of pancreatic cancer [15-18]. Celecoxib, a highly selective COX-2 inhibitor, can induce tumor cell apoptosis and inhibit the proliferation of a variety of solid tumors including pancreatic cancer; in addition, it can enhance the lethal effects of many chemotherapy drugs on cancers [19]. Our previous studies have shown that celecoxib can effectively inhibit the growth and endothelial angiogenesis of human HepG2 cell xenografts in nude mice, by inhibiting the expression of COX-2 [20]. Further studies confirmed that α-interferon and the COX-2 inhibitor celecoxib synergistically increased TRAIL expression in hepatocellular carcinoma to induce apoptosis [21].

However, the clinical benefits and the mechanism of S-1 in combination with celecoxib for the treatment of pancreatic cancer are not clear. The aims of the present study were to investigate the synergistic role of S-1 and celecoxib in pancreatic cancer growth and to explore the underlying mechanisms.

Methods

Cell lines and animals

The human pancreatic cancer cell line, PANC-1, was supplied by the Cell Resource Center of Chinese Academy of Sciences Shanghai Institutes for Biological Sciences; Forty male BALB/C nude mice (6-week-old, weighing 10~20 g) were purchased from Hunan Slack King Laboratory Animal (Changsha, China) with animal certificate No. SYXK (Jing) 2013-0004.

Compounds and reagents

Celecoxib was purchased from Pfizer Inc. (New York, USA) with the approval number BK11-CCEE042, dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C; S-1 was delivered from Shandong New Time Pharmaceutical (approval number: H20080802), dissolved in PBS and stored at -20°C; DAPI staining solution and RIPA lysis buffer were bought from Beyotime Institute of Biotechnology (Haimen, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA); Apoptosis detection kits were purchased from KeyGEN BioTECH (Nanjing, China); Monoclonal antibodies to Bcl-2 (ab117115), caspase-3, Bax (ab32503), PARP (ab6079), survivin (ab-182132) and β-actin were obtained from Santa-Cruz Biotechnology (Santa-Cruz, CA, USA); phosphate-buffered saline (PBS), DMEM medium, fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were purchased from Gibco (Grand Island, NY, USA); PCR kit, Premix, Ex Taq and Trizol were sourced from Wuhan Boster...
Clinical benefits and actions of S-1 in combination with celecoxib

Biological Technology (Wuhan, China); Primer was obtained from Shanghai Biological Engineering (Shanghai, China); 0.25% trypsin was bought from Hyclone Laboratories (Logan, UT, USA), PrimeScript\textsuperscript{TM} RT reagent Kit was supplied by TaKaRa Bio Inc. (Kusatsu, Japan).

Cell culture and treatments

PANC-1 cells were cultured in completed DMEM medium, containing 10% FBS, 20 mmol/L NaHCO\textsubscript{3}, 100 U/mL penicillin and 100 µg/ml streptomycin, at 37°C in a 5% CO\textsubscript{2} atmosphere incubator. PANC-1 cells were treated with various concentrations of S-1, celecoxib or both for the indicated times.

Cell viability assay

Cell viability was analyzed by MTT assay. Briefly, PANC-1 cells in the logarithmic phase were seeded at a density of 1 × 10\textsuperscript{5} cells/100 µL/well, in 96-well plates, and incubated at 37°C overnight for attachment, in a 5% CO\textsubscript{2} atmosphere incubator. Then the PANC-1 cells, in the S-1 group were challenged with 0, 5, 10, 20 μM of S-1; the celecoxib group were challenged with 0, 20, 40 and 80 μM celecoxib; the S-1 and celecoxib combination group were challenged with 0, 5, 10 or 20 μM S-1 with 40 μM of celecoxib, respectively. After 24 h, 20 μL MTT (5 mg/mL) was added and cultured at 37°C for 4 h. Then the supernatant was discarded and 150 μL DMSO was added to each well and incubated at room temperature for 10 min. Absorbance was read at 490 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). Four wells were analyzed at each concentration. Each experiment was repeated 3 times. The percentage of cell viability was calculated according to the following equation: Cell viability (%) = OD\textsubscript{treated}/OD\textsubscript{control} × 100%. The amount of DMSO used in this study has been proven not to affect the proliferation of pancreatic cancer cells (Data not shown). The average cell viability is presented as the mean ± standard deviation (SD). The synergetic effects of S-1 and celecoxib were evaluated by the Q value method [22].

Analysis of the cell cycle and apoptosis using flow cytometry

Logarithmic phase PANC-1 cells were seeded at a density of 5 × 10\textsuperscript{5} cells/2 mL/well, in 6-well plates and incubated at 37°C overnight in a 5% CO\textsubscript{2} atmosphere to facilitate attachment. The supernatant was replaced with fresh DMEM medium. PANC-1 cells were then challenged with PBS in the control group, 40 μM celecoxib in the celecoxib group and 10 μM S-1 in the S-1 group, 40 μM celecoxib and 10 μM S-1 in the S-1 and celecoxib combination group, respectively. Harvested PANC-1 cells (1 × 10\textsuperscript{5}) were washed twice with pre-cooled PBS and re-suspended in 1 mL of pre-cooled 70% ethanol and fixed at 4°C for 24 h. The ethanol was discarded by centrifugation and cells washed with ice-cold PBS. The PANC-1 cells were then re-suspended in 500 µL of 1 × binding buffer containing 100 µg/mL PI (for the cell cycle assay) or 100 µg/mL PI and Annexin V-FITC mixture (for the apoptosis assay), and samples left in the dark for 30 min. The apoptosis rate was analyzed by FACS Calibur flow cytometry (Beckman Coulter EPICS XL, CA, USA). The early apoptotic cells (Annexin-V+/PI-) were distributed in the lower right quadrant. The late apoptotic cells (Annexin-V+/PI+) were distributed in the upper right quadrant. The lower left quadrant indicates normal cells and the lower right quadrant represents early apoptotic cells (Annexin-v- and PI-).

The cell cycle distribution is shown as the percentage of cells containing G\textsubscript{0}/G\textsubscript{1}, S and G\textsubscript{2}/M DNA content, as determined by propidium iodide staining.

Western blot analysis

PANC-1 cells in 6-well plates were pretreated with PBS in the control group, 40 μM celecoxib in the celecoxib group, 10 μM S-1 in the S-1 group or 40 μM celecoxib and 10 μM S-1 in the S-1 and celecoxib combination group, and washed twice with cold PBS. PANC-1 cells were resuspended in RIPA cell lysis buffer containing proteinase inhibitors (100:1) and kept on ice for 15 min with scratch. The supernatant of the cell lysate was collected by centrifugation at 13,000 g, 4°C for 5 min. The protein concentration was determined by the BCA method according to the manufacturer’s description. 25 µg of proteins were run on 12% SDS-PAGE gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (120 V × 90 min). Membranes were blocked with 5% non-fat milk for 1 h at room temperature, and then
incubated overnight at 4°C with primary antibodies of survivin, Bcl-2, caspase-3, PARP and Bax (1:1000 dilution). Signals were developed using an enhanced chemiluminescent (ECL) detection kit (Pierce, Rockford, IL, USA) after incubation with peroxidase-conjugated secondary antibodies for 2 h at room temperature.

**Fluorescence microscope observation**

PANC-1 cells were seeded in 12-well culture plates at a density of 5 × 10^5 cells/mL/well and cultured for 12 h. These cells were then challenged with PBS in the control group, 40 μM celecoxib in the celecoxib group, 10 μM S-1 in the S-1 group, 40 μM celecoxib and 10 μM S-1 in the S-1 and celecoxib combination group, respectively, for 24 h. The supernatant was discarded by centrifugation at 1,000 r/min for 15 min and the cells washed once with PBS before being fixed with methanol/acetic acid solution (3:1) for 10 min. The cells were re-suspended in a small amount of the fixing solution and a drop of the cell suspension placed on a cover slip. Cells were then stained with DAPI for 10 min and left to naturally dry and then rinsed with distilled water and mounted in fluorescent solution (a mixture of equal volumes of PBS and glycerol, pH 9.5). The cells were viewed and photographed under a fluorescence microscope at a 360~400 nm excitation wavelength.

**In vitro cell migration assay**

Migration of PANC-1 cells was determined by the wound-healing assay. Briefly, logarithmic phase PANC-1 cells were seeded at a density of 1 × 10^4 cells/mL/well, in 6-well plates. The cells were pretreated (vide supra) for the indicated groups. A straight line was marked using a pencil on the back of the wells containing pretreated cells. A straight line, perpendicular to the one on the back of each well at the center, was gently scraped using the tip of a dispos-able pipette and then the pancreatic cancer cells were grown to 95% confluence. The wells were then rinsed with sterile PBS 3 times and recharged with serum-free DMEM medium. Wound healing was observed and photographed after 48 h.

**Cell-matrix adhesion assay**

The 96-well culture plates were coated by adding the diluted (1:5 dilution) artificial Matrigel (100 μL/well) at a final concentration of 100 ng/mL. Logarithmic phase PANC-1 cells were seeded at a density of 5 × 10^4 cells/well into the coated plates, after being kept at 37°C in an oven for 1 h before being incubated at 37°C in a 5% CO₂ atmosphere incubator for 12 h. The PANC-1 cells were then challenged with PBS in the control group, 40 μM celecoxib in the celecoxib group, 10 μM S-1 in the S-1 group, 40 μM celecoxib and 10 μM S-1 in the S-1 and celecoxib combination groups, respectively. Challenged cells were cultured for 24 h in triplicates for each concentration. MTT solution (5 mg/ml) was added (20 μl/well) and cultured for another 4 h. The supernatant was discarded and replenished with 150 μL of DMSO in each well. The plates were oscillated for 10 min on an automatic shaker in a low-speed mode. Absorbance (A value) was measured at a wavelength of 490 nm using a microplate reader. Relative adhesion rate% = (A_490 value of the experimental group/A_490 value of the control group) × 100%. The above experiments were repeated 3 times.

**RNA purification and quantitative real-Time PCR (qRT-PCR) analysis**

Pretreated PANC-1 cells in PBS for the control group, 40 μM celecoxib in the celecoxib group, 10 μM S-1 in the S-1 group, or 40 μM celecoxib and 10 μM S-1 in the combination therapeutic group, were washed twice with cold PBS. Total RNA was isolated using Trizol reagent. Briefly, cells in each well were lysed with 1 mL of Trizol at the end of the intervention and collected into 1.5 mL Eppendorf tubes. After being washed once with PBS, the tubes were shaken after 200 μL of chloroform was added to each tube and then 400 μL of the upper liquid aqueous phase carefully aspirated into fresh Eppendorf tubes, after centrifugation at 12,000 rpm for 15 min at 4°C. Isopropyl alcohol (500 μL) was added to each tube, mixed and tubes were then left on ice for 10 min; then the supernatant was discarded after centrifugation at 12,000 rpm for 15 min at 4°C. RNA was naturally dried at room temperature for < 10 min after washing with 75% ethanol. The precipitated RNA was fully dissolved in 200 μL of DEPC pretreated H₂O. RNA concentrations were measured using a spectrophotometer; the OD_{260}/OD_{280} ratio of the samples should have been between 1.8-2.0. cDNA synthesis was conducted using a PrimeScript™ RT reagent kit (Takara Bio Inc., Japan) according to...
the manufacturer’s protocol. Briefly, a total of 10 μL mixture, including 2.0 μL 5 × g DNA Eraser Buffer, 1.0 μL g DNA Eraser, 1.0 μg total RNA and DEPC treated H2O, was added to sample tubes which were placed in a 42°C water bath for 2 min. Then, 1.0 μL of RT Primer Mix, 4.0 μL 5 × PrimeScript Buffer 2 and 5.0 μL DEPC treated H2O were added into the mixture prepared above on ice to a final volume of 20 μL. After brief centrifugation, PCR was carried out using a Real-time PCR system (Bio-Rad, CA, USA) under the conditions of 40 cycles with pre-denaturation at 95°C for 5 min, denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec and elongation at 72°C for 20 sec. The primers used were: GAPDH: forward (F), 5′-TTTG-TCAAGCTCAT TTCTCTG-3′; reverse (R), 5′-TGGTCCAGGGTT-TCTTACT-3′; survivin: F, 5′- TTCTCAAGGACCACCGC-3′; R, 5′-GCCAAGTCTGCTCG-TTCT-3′; COX-2: F, 5′-CTCTC-AATGAGTACCGCAAA-3′; R, 5′-CAAAGATAGCATCTGCCGAGA-3′.

**Table 1.** Influence of S-1 combined with celecoxib on the inhibition rate of PANC-1 cell viability

<table>
<thead>
<tr>
<th>Groups (n = 4)</th>
<th>Concentration</th>
<th>Inhibition rate (%) of cell proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Celecoxib group</td>
<td>20 μmol/L</td>
<td>27.31 ± 1.77</td>
</tr>
<tr>
<td></td>
<td>40 μmol/L</td>
<td>32.80 ± 4.85</td>
</tr>
<tr>
<td></td>
<td>80 μmol/L</td>
<td>57.89 ± 7.93</td>
</tr>
<tr>
<td>S-1 group</td>
<td>5 μmol/L</td>
<td>20.08 ± 9.11</td>
</tr>
<tr>
<td></td>
<td>10 μmol/L</td>
<td>31.14 ± 11.15</td>
</tr>
<tr>
<td></td>
<td>20 μmol/L</td>
<td>54.56 ± 7.72</td>
</tr>
<tr>
<td>Combination group</td>
<td>5 μmol/L S-1 + 40 μM celecoxib</td>
<td>63.08 ± 19.15**</td>
</tr>
<tr>
<td></td>
<td>10 μmol/L S-1 + 40 μM celecoxib</td>
<td>72.11 ± 6.97**</td>
</tr>
<tr>
<td></td>
<td>20 μmol/L S-1 + 40 μM celecoxib</td>
<td>88.09 ± 17.22**</td>
</tr>
</tbody>
</table>

Note: Influence of S-1 combined with celecoxib on the inhibition rate of PANC-1 cell viability. PANC-1 cells were challenged with different concentrations of S-1, celecoxib or S-1 combined with celecoxib for 24 h, followed by MTT assay. Data are representative of 3 independent experiments. *P < 0.05 compared to the next lower dose. **P < 0.01 compared to monotherapy.

**Figure 1.** Percent distribution for specific phases across the PANC-1 cell cycle. After treatment for 24 h, the effects of S-1 (10 μM), celecoxib (40 μM) or S-1 combined with celecoxib on the cell cycle of PANC-1 cells were determined by flow cytometry. All the experiments were repeated 3 times. *P < 0.05.

Tumor growth assay (Tumor Xenograft in nude mice)

Forty (six-week-old) male athymic (nu/nu) mice were used in the study, with the approval of the Institutional Animal Care and Use Committee of the University. 250 μL (2 × 10⁷/mL) of PANC-1 cells in PBS were injected subcutaneously under the front legs with a total final number of 5 × 10⁶ administered per mouse. The mice were observed every day and the long diameter (a) and short diameter (b) of tumors were measured with a caliper every 3 days to monitor tumor development. All of the mice bearing tumors were randomly separated into a negative control group, a celecoxib group, a S-1 group and a S-1 and celecoxib combination group, when the long diameter of the tumor reached 5 mm. Celecoxib and S-1 were administered to mice intragastrically after the drugs were suspended in sodium carboxymethyl cellulose (5 g/L). The dose of celecoxib in the celecoxib group was 50 mg/kg·d [23], with continuous administration carried out for 7 d; the dose of S-1 in the S-1 group was 8.3 mg/kg·d [24], with continuous administration for 7 d; the doses and frequencies of celecoxib and S-1 in the S-1 and celecoxib com-
Clinical benefits and actions of S-1 in combination with celecoxib

Immunohistochemistry assay

For immunohistochemistry, 20 mg of tumor tissue was fixed in 10% neutral buffered paraformaldehyde at 4°C for 24 h. Sections (5-μm thick) were deparaffinized, rehydrated with PBS (pH 7.4), treated with aqueous 3% H2O2 for 10 min and the antigen retrieved in 0.1% trypsin (M/V) for 10 min at 37°C. Sections were blocked with 5% BSA at room temperature for 30 min, then rabbit anti-survivin antibody was applied at a concentration of 1:150 overnight at 4°C. Then they were incubated for 15 min at room temperature with a second antibody conjugated with HRP after washing with PBS. Diaminobenzidine (DAB) was applied for 5 min and then slides were counterstained with hematoxylin. Sections were then mounted with neutral gum after being made transparent with xylene. Images were acquired using an optical microscope (Olympus IX70, Japan). The extent of survivin-positive expression, which was localized in the cytoplasm of tumor cells, was calculated using Image-Pro Plus.

Statistical analysis

SPSS Statistics for Windows software (ver. 18.0, SPSS Inc., Chicago, US) was used for all statistical analyses. Data are representative of 3 independent experiments. Means ± standard deviation (SD) are shown. PCR results were analyzed using BandScan analysis software. The synergetic effects of the test drugs were evaluated by the Q value method [22], according to the following equation: $q = \frac{E_{A+B}}{E_A + E_B} - E_A \cdot E_B$ ($E_A$ represents the effect of the S-1 intervention; $E_B$ represents the effect of the celecoxib intervention; $E_{A+B}$ represents the effect of S-1 with celecoxib. The q value: 0.85 to 1.15 means a simple addition; 1.15 to 20 means augmentation; values > 20 means significant augmentation; 0.85 to 0.55 means antagonism; values < 0.55 means obvious antagonism. Completely randomized design ANOVA was performed to compare the difference between groups. P-values < 0.05 were considered to be statistically significant.

Results

S-1 and celecoxib synergistically inhibited the viability of human pancreatic cancer PANC-1 cells

After being challenged with different concentrations of S-1, celecoxib or S-1 combined with celecoxib for 24 h, PANC-1 cell viabilities were quantified by MTT assay. As shown in Table 1, S-1 or celecoxib had a significant dose-dependent inhibitory effect on the viability of PANC-1 cells. However, the inhibition rates of S-1 or celecoxib were lower than in the S-1 and celecoxib combination group. The inhibition rate of S-1, at a final concentration of 20 μM, was only 54.56%. Differences between the inhibitory effects of S-1 and celecoxib were not statistically significant ($P > 0.05$). The inhibitory effects were more obvious when PANC-1 cells were challenged with different concentrations of S-1 combined with celecoxib (40 μM). The inhibition rates of S-1 combined with celecoxib on the PANC-1 cell viability were significantly increased vs the control group or each monotherapy group ($P < 0.05$), which were gradually
improved with an increase in the S-1 concentration. The inhibition rate was 88.09% when 20 μM S-1 and 40 μM celecoxib were used in combination. The calculated q = 1.325 was higher than 1.15, which indicated that S-1 and celecoxib had synergistic inhibitory effects on the viability of PANC-1 cells.

S-1 and celecoxib synergistically inhibit the cell cycle progression of PANC-1 cells

After being challenged with S-1 (10 μM), celecoxib (40 μM) or S-1 combined with celecoxib for 24 h, the cell cycle phases of PANC-1 cells were evaluated by flow cytometry. The challenge resulted in G0/G1 phase arrest, and a corresponding reduction in the G2/M and S phases of PANC-1 cells, which was most obvious in the combination group (Figure 1). The difference of each treatment group vs the control group was statistically significant (P < 0.05); differences in the proportion of each cell phase between the S-1 and celecoxib combination group and each monotherapy group were also statistically significant (P < 0.05). These data suggested that
S-1 and celecoxib may synergistically inhibit PANC-1 cell survival by inhibiting proliferation, due to arrest of the cell cycle and DNA synthesis.

S-1 and celecoxib synergistically induced apoptosis of PANC-1 cells

After being challenged with S-1 (10 μM), celecoxib (40 μM) or S-1 combined with celecoxib for 24 h, apoptosis of PANC-1 cells was analyzed by flow cytometry and fluorescence microscopy. As shown in Figure 2, 10 μM S-1 combined with 40 μM celecoxib could significantly promote PANC-1 cell apoptosis compared with the control group (26.38% vs 4.40%).

We further checked the morphological changes in apoptotic cells after DAPI staining using fluorescence microscopy. As illustrated in Figure 3, PANC-1 cells in the control group had intact cell membranes with deep colored, full shaped and uniform density nuclei (Figure 3A). After being challenged with S-1 (10 μM) and/or celecoxib (40 μM), cell membrane shrinkage, relatively small nuclei, chromatin concentration and fluorescence color enhancement could be seen in some of the cells. Apoptotic bodies, with the typical apoptotic morphology, could be seen in some fields of view, which were characterized by condensed cells with unequal sizes of cellular debris (Figure 3B-D). The morphological changes representing apoptosis was most obvious in the combination group (Figure 3D). These results further demonstrated that S-1 and celecoxib could synergistically promote apoptosis of pancreatic cancer PANC-1 cells.

S-1 and celecoxib synergistically regulated the expression of apoptosis-related factors

S-1 (10 μM) and celecoxib (40 μM) treatment inhibited the expression and transcription of the anti-apoptotic proteins Bcl-2 and survivin, while increasing the expression of the pro-apoptotic protein Bax (Figure 4A and 4C). Subsequently, the intrinsic caspase signaling was triggered, with caspase-3 and caspase-8 being statistically significantly upregulated (Figure 4B), as well as enhanced proteolytic cleavage of PARP (Figure 4A). Figure 4A shows the analy-
Clinical benefits and actions of S-1 in combination with celecoxib

The effects of S-1 (10 μM) and celecoxib (40 μM) on PANC-1 cell migration and invasion were examined using the wound healing assay and the cell-matrix adhesion assay, respectively as described in the Materials and Methods section. S-1 and celecoxib significantly suppressed PANC-1 cell migration (Figure 5A-D) and invasion (Figure 5E), with S-1 combined with celecoxib exhibiting the best inhibitory effect. The inhibitory effect on PANC-1 cell invasion had a dose-dependent relationship; the differences between the S-1 and celecoxib combination as well as the S-1 and celecoxib groups and the control group were statistically significant ($P < 0.05$); while between the S-1 and celecoxib group no statistically significant difference was obtained ($P > 0.05$).

S-1 and celecoxib synergistically reversed cell migration and invasion of PANC-1 cells

Six days after PANC-1 cell inoculation, 2 of the 40 mice failed to form tumors (long diameter tumor ≥ 5 mm), with a tumor formation rate of 95%. Celecoxib (50 mg/kg·d) and S-1 (8.3 mg/(kg·d) were administered to mice. After intragastric dosing of S-1 and/or celecoxib, the mice did not show significant adverse reactions; mental condition, activity, diet and defecation of mice were all normal. Body weight increased after the treatment. Mice were sacrificed after continuous administration for 27 days and tumors were isolated. The tumor volumes of subcutaneous xenografts in each group were: control group (2,288.67 ± 753.87) mm$^3$, S-1 group (1,542.42 ± 229.17) mm$^3$, celecoxib group (1,383.75 ± 323.25) mm$^3$, and S-1 and celecoxib combination group (615.00 ± 195.25) mm$^3$ (Figure 6). Tumor growth was slower, thus the tumor volume was significantly reduced ($P < 0.05$) in each treatment group vs the control group; tumor volume in the S-1 and celecoxib combination group was significantly smaller than in each monotherapy group ($P < 0.05$); tumor volumes in the S-1 group and celecoxib group exhibited no significant differences ($P > 0.05$). Inhibition rates of tumor growth were 32.61%, 39.53% and 73.13% for the S-1 group, celecoxib group and the S-1 combined with celecoxib group, respectively. The $q = 1.23 > 1.15$ indicated that S-1 and celecoxib produced significant synergistic effects when used in combination.

S-1 and celecoxib synergistically inhibited the expression of survivin in vivo

To confirm further the inhibitory effects of S-1 (8.3 mg/kg·d) and celecoxib (50 mg/kg·d) on the expression of survivin in vivo, we investigated the expression of survivin in xenograft tumor tissue by immunohistochemical staining. As shown in Figure 7, compared with the con-
Clinical benefits and actions of S-1 in combination with celecoxib

trol group, S-1 and celecoxib significantly reduced survivin expression in xenograft tumor tissue ($P < 0.05$); compared with the mono-therapy group, survivin expression in xenograft tumor tissue was significantly reduced in the S-1 and celecoxib combination group ($P < 0.05$); while survivin expression in the S-1 group xenograft tumor tissue was greater than in the celecoxib group ($P < 0.05$).

Discussion

Gemcitabine has been the first-line clinical treatment for pancreatic cancer over the past 10 years. Even though the outcome of single-agent gemcitabine treatment against pancreatic cancer is limited, other remedies alone or in combination failed to demonstrate a survival benefit greater than gemcitabine monotherapy alone, until the combination of gemcitabine and erlotinib was shown to bring significant benefits [25, 26]. Later, it was reported that FOLFIRINOX (a combination chemotherapy regimen including fluorouracil, oxaliplatin, irinotecan and leucovorin) provided a statistically and clinically significant advantage over gemcitabine monotherapy in advanced pancreatic cancer patients. This is the first actual therapeutic progress achieved since the introduction of gemcitabine even though the toxicity associated with the S-1 and celecoxib combination is significantly increased. It is strongly recommended to use the combination treatment as a more effective strategy to improve outcomes in patients with advanced pancreatic cancer than gemcitabine monotherapy alone [27].

Apoptosis is a physiological process of programmed cell death that removes cells no longer required or that have become irreparably damaged. Abnormal regulation of apoptosis is an important mechanism in cancer pathogenesis [28], and has been widely recognized as a hallmark of most types of cancer [29]. By regulation of specific mechanisms that are usually involved in triggering cancer cell apoptosis, drugs that can induce cancer cell apoptosis hold great promise for therapy and have been the focus of many preclinical drug discovery studies. Apoptosis is initiated by cell-surface death receptors such as Fas (the extrinsic pathway) after directly activating a caspase-activation cascade. The mitochondrial pathway (the intrinsic pathway) of apoptosis, on the other hand, is triggered by loss of the integrity of the mitochondrial outer membrane, which allows the release of pro-apoptotic factors (e.g., cytochrome C) from the mitochondria into the cytosol [30, 31]. In our study, for the first time, it has been demonstrated that S-1 and celecoxib can synergistically inhibit the viability of human...
pancreatic cancer PANC-1 cells in a dose-dependent manner. To identify further the specific apoptosis pathways involved in S-1 and celecoxib-induced PANC-1 cell apoptosis, the expression of caspase-3 and caspase-8 were investigated and the results revealed that both caspase-3 and caspase-8 were upregulated in PANC-1 cells after exposure to S-1 and celecoxib, strongly suggesting that the extrinsic pathway for apoptosis had been triggered.

Survivin is by far one of the strongest apoptosis inhibitor proteins and an important indicator of tumor proliferation and malignancy [32]. Survivin can directly inhibit the activity of apoptosis terminal effector enzyme caspase-3 and caspase-7, and simultaneously interfere with the activity of caspase-9, thereby suppressing Fas or TRAIL receptor-induced apoptosis. Survivin can also cause ribosome phosphorylation by binding to the cell cycle regulator CDK4, thus accelerating the cell cycle G1/S conversion process, thus indirectly inhibiting the activity of caspase-3 by the release of P21 and preventing mitochondrial release of cytochrome C, thereby inhibiting apoptosis [33]. In the present we found that S-1 and celecoxib could inhibit the expression of survivin in both human pancreatic cancer PANC-1 cells and xenograft tumor tissues at the mRNA and protein level, in a dose-dependent manner. Cell cycle analysis indicated that combination treatment with S-1 and celecoxib resulted in a statistically significant G0/G1 phase arrest of PANC-1 cells, which in combination with enhanced apoptosis reflected essentially suppressed tumor growth in xenograft-bearing nude mice after S-1 plus celecoxib application with a q value of 1.23, indicating that both drugs acted synergistically. Taken together, our data suggests that S-1 and celecoxib synergistically inhibited PANC-1 cell growth in vitro and in vivo, by inhibiting proliferation due to cell cycle arrest and induced apoptosis, shown by downregulation of Bcl-2 and survivin as well as upregulation of Bax. Our findings provide a theoretical basis for the use of S-1 combined with celecoxib as a new therapeutic strategy to treat human pancreatic cancer.

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Disclosure of conflict of interest

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References

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