HERG K⁺ channel related chemosensitivity to sparfloxacin in colon cancer cells

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Abstract. Potassium channels are essential for the regulation of cell proliferation. As reported, HERG protein is overexpressed in a wide range of human tumors, including colon carcinoma. The aim of this study was to investigate the effects of antibacterial agents sparfloxacin (SPFX), a blocker of HERG channel, on HERG K⁺ channel highly expressing colon cancer cells. Expression of HERG and apoptosis correlative proteins was examined by Western blotting. The MTT assay was used to detect the cytotoxicity of drugs and drug combination in vitro. Gene transfection was used to examine the changes in herg-related chemosensitivity. Cell apoptosis was analyzed by flow cytometry. The migration and invasion capacity of tumor cells by SPFX was determined by gelatin zymography assay and Boyden chamber. The in vivo efficacy of SPFX was assessed in murine colon carcinoma C26 in BALB/c mice and human colon carcinoma HCT116 xenografts in nude mice. High expression of HERG protein was detected in colon cancer C26, HCT116 and HT-29 cells. The cell viability of the colon cancer cells was inhibited by SPFX in a dose-dependent manner. SPFX induced apoptosis and inhibited migration and invasion of colon cancer HCT116 cells. The increase in apoptosis was associated with a decrease in procaspase-3 and Bcl-2 protein expression. Study with herg-transfected HEK293 cells and siRNA-knock down HCT116 cells confirmed that the cell viability inhibition by SPFX was correlated with HERG expression. When combined with 5-fluorouracil, SPFX showed synergistic anti-proliferation activity in HCT116 and HT-29 cells. Furthermore, SPFX inhibited the growth of human colon carcinoma HCT116 xenografts and showed synergistic effect with 5-fluorouracil in vivo. Our finding suggested that SPFX could be a biochemical modulator in treatment of colon cancer with chemotherapeutic drugs.

Introduction

Colorectal cancer is one of the major causes of cancer death worldwide. Chemotherapy remains the major treatment of advanced colorectal cancers. The advent of oxaliplatin and irinotecan combined with 5-fluorouracil/leucovorin (5-FU/LV) proved to significantly increase overall response rates and survival times (1). Intense efforts have focused on identifying new therapeutic strategies, which included novel agents targeting, specific growth factor receptors, critical signal transduction pathways or mediators of angiogenesis. In addition, drug combinations that may display synergy and lead to improvement of efficacy have attracted much attention. Several clinical trials have suggested that some of the molecular targeted drugs can be safely and effectively used in combination with conventional chemotherapy (2).

The herg (human ether-a-go-go-related gene) belongs to an evolutionarily conserved multigenic family of voltage-activated K⁺ channels, the eag (ether-a-go-go) family (3). HERG is mainly expressed in the cell membrane of cardiac myocytes and the role of HERG in myocardium cell is best understood, where it contributes to the repolarization of the cardiac action potential (4,5). Recently, HERG has been found overexpressed in many human tumor cell lines and tumors of distinct histogenesis (6). Studies revealed that HERG protein is selectively upregulated in a variety of human and animal tumors while its expression is absent in the normal tissue or cell line counterparts (7,8). Moreover, selective pharmacological blockage of the HERG channel in several tumor cell types significantly reduced the cell proliferation (8-10). One possibility is that the existence of HERG channel contributes to maintain a more depolarized membrane potential and thus permits an easier passage through the cell cycle (11). In addition, other studies demonstrated that the expression of the HERG protein was cell cycle-dependent and physically interacted with TNFR (tumor necrosis factor receptor) in the cell membrane of tumor cell lines (12,13). The HERG protein also regulates cell invasion of colon cancer cells and modulates VEGF secretion in glioblastoma cells (14,15). Data gathered thus far suggest that HERG channels may play a prominent role in the control of tumor cell proliferation and apoptosis.

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HERG channel may be a useful target for cancer therapy. Our previous studies have found that HERG expression level may relate to the chemosensitivity in cancer cells and erythromycin, the blocker of HERG channels, may modulate HERG related chemosensitivity (16,17).

The quinolones are agents that have broad-spectrum and potent antibacterial activity. Furthermore, quinolones prove to be not only useful antibacterial agents, but also exert immunomodulation and antitumor effects (18,19). Sparfloxacin (SPFX) is one of the fluoroquinolone antibacterials and known to inhibit the HERG channel. Using patch-clamp electrophysiology testing inhibition of HERG channel currents, SPFX was the most potent compound among the fluoroquinolones (20-22). In lab research, SPFX can reverse drug resistance via P-glycoprotein (23). SPFX exhibits antitumor activity under ultrasonic irradiation and can be as potentially valuable sonodynamic compound (24). As both HERG and its blocker-SPFX, an antibacterial agent have been implicated in antitumor processes, it is of great interest to further investigate the effects of SPFX on HERG highly expressing cancer cells and its modulation on the efficacy of anticancer drugs. We examined the effects of SPFX on cell viability and apoptosis, and to determine if there is any synergistic antitumor effect of SPFX in the combination of antitumor chemotherapeutic agents in vitro or in vivo.

Materials and methods

Cell culture and drugs. Human embryonic kidney HEK293 cells were grown in DMEM containing 10% FCS and 100 IU/ml penicillin and 100 μg/ml streptomycin. Human colon carcinoma cell lines (HT-29 and HCT116), and murine colon carcinoma C26 cell line were cultured in RPMI-1640 and incubated at 37°C in a humidified atmosphere with 5% CO₂. SPFX (Sigma) was prepared as a 50 mmol/l stock solution in 0.1 N NaOH. 5-fluouracil (from Shanghai Xudong Haipu Pharmaceuticals, China) was prepared as 1 mmol/l stock solutions in PBS. The drugs were diluted in fresh DMEM without serum before each experiment and added to culture medium for a final concentration.

HERG protein extraction and Western blot analysis. For whole cell proteins, the treated cells were lysed in 100-200 μl RIPA buffer (50 mmol/l Tris pH 8.0, 150 mmol/l NaCl, 1 mmol/l EDTA, 2 mmol/l Na₃VO₄, 1% NP-40, 10 mmol/l PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin). The protein content was measured using the BCA™ Bradford protein assay (Pierce, USA). Protein extracts were boiled for 5 min in 6X loading buffer and then equal amounts of total proteins were separated by 7.5-10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were decorated with the primary antibody and then with HRP-conjugated secondary antibody. Detection was carried out using an enhanced chemiluminescence agent (Millipore Corporation, Billerica, MA). Actin served as an internal control.

Antibody to HERG was purchased from Chemicon International Inc. (AB5908; Chemicon, USA). Antibodies to procaspase-3, Bel-2, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Gene transfection. HERG cDNA (from Dr Ge-Nya Tseng, Virginia Commonwealth University, USA) was subcloned into BamHI/HindIII sites of the pCDNA3.1 vector (Invitrogen). U6pro siRNA plasmids (from Dr J.Q. Zheng, Beijing Institute of Pharmacology and Toxicology, Beijing, China) were purified using Wizard PureFecction plasmid DNA purification system (Promega, Madison, WI).

Cells were plated in 96-well plates or in 6-well dishes. When cells grew to 90% confluence, the transfection of the plasmid was performed using lipofectamine 2000 method (Invitrogen). For MTT assays, after 8 h transfection, different concentrations of drugs were added. Experiments were repeated at least three times. For Western blotting, cells were seeded in 6-well plates and the membrane proteins were collected after 48 h transfection.

MTT assay. Cells were detached by trypsinization, seeded at 3,000-6,000 cells/well in 96-well plate (Costar, Cambridge, MA) overnight. Then the test compounds were added and incubated for a further 48 h. The effect on cell growth was examined by MTT assay. MTT solution (20 μl) (5 mg/ml) were added to each well and incubated at 37°C for 4 h. The supernatant was removed, and the MTT formazan formed by metabolically viable cells was dissolved in 150 μl of DMSO, and then monitored with a microplate reader (Bio-Rad) at a wavelength of 570 nm.

The inhibition rate was calculated according to the formula: inhibition rate (%) = (absorbency of control - absorbency of treated cells)/absorbency of control x100.

Coefficient of drug interaction (CDI) was calculated according to the formula: CDI = AB/(AxB), where A and B are the survival values (in vitro) or weight of tumor (in vivo) with respective single agent and AB is the survival values or weight of tumor (in vivo) of two drugs combination (25,26). A synergistic effect was considered to be a two-drug combination for CDI <1 and significantly synergistic effect of a two-drug combination for CDI <0.7.

Flow cytometry analysis of apoptosis. Percentage of sub-G1 was determined by flow cytometry. Cancer cells were seeded in 6-well plates and exposed to various drugs for 48 h. Cells were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 75% ethanol at 4°C overnight. Subsequently, the fixed cells were incubated in a solution containing 100 μg/ml RNase for 30 min and then 50 μg/ml propidium iodide for 30 min at 37°C in the dark. The cells were then analyzed by FACSCan (Beckman Coulter). Cell apoptosis was measured according to the protocol of Annexin V-FITC/PI apoptosis detection kit.

Wound closure assay. HCT116 cells were seeded at 1x10⁴ cells/well in 96-well plates. After cells reached 90% confluence, a wound was incised with a pipette tip in the central area of culture, and SPFX was added. Photographs were taken under microscope immediately after the incision and after 36 h of SPFX treatment.

Cell migration and invasion assays. To assess cell motility, chamber assays were performed using transwell cell migration chamber plates (8-μm pore size, 12-well format; Corning,
was initiated when the tumor volume reached about 100 mm³. Treatment cells/mouse. A tumor piece of 2-3 mm in diameter was
remove SDS, subsequently transferred to a buffer (50 mmol/l
washed in 2.5% Triton X-100 for 1 h at room temperature to
(Invitrogen, Carlsbad, CA). After electrophoresis, gels were
for another 24 h. Then 20 μg proteins from each supernatant
concentrations of SPFX 24 h and RPMI-1640 without serum
cells/well in 24-well plates and then treated with varying
incubation with varying concentrations of SPFX at 37˚C,
chamber. The lower compartment of the chamber was filled
trations of SPFX and placed in the upper compartment of the
administered twice a day for 20 days. 5-FU was injected
i.g. (intragastric gavage) with saline (as control) and SPFX
were sacrificed and tumor weight was measured.
USA). HCT116 cells (2x10⁶ cells) were suspended in 100 μl
RPMI-1640 containing 1% serum with varying concen-
trations of SPFX and placed in the upper compartment of the
chamber. The lower compartment of the chamber was filled
with 600 μl RPMI-1640 containing 20% serum. To assess
cell invasion, total 50-μl matrigel was dispersed on the upper
side of the transwell cell migration chamber. After 24 h
of incubation with varying concentrations of SPFX at 37˚C,
the non-migratory or non-invasive cells were removed with a
cotton-tipped applicator, and the cells that penetrated through
to the bottom of the chamber were stained with hematoxylin.
The cells that had invaded through Matrigel and reached the
reverse side were counted under a microscope in five pre-
determined fields at a magnification of x400. Each assay was
performed in triplicate.

**Gelatin zymography.** HCT116 cells were seeded at 2x10⁴
cells/well in 24-well plates and then treated with varying
concentrations of SPFX 24 h and RPMI-1640 without serum
for another 24 h. Then 20 μg proteins from each supernatant
were applied in 2X Tris-Glycine gel loading buffer and electro-
phoresed in 10% SDS-PAGE gels containing 0.1% gelatin
(Invitrogen, Carlsbad, CA). After electrophoresis, gels were
washed in 2.5% Triton X-100 for 1 h at room temperature to
remove SDS, subsequently transferred to a buffer (50 mmol/l
Tris, 10 mmol/l CaCl2, 200 mmol/l NaCl, 1 μmol/l ZnCl₂,
pH 7.5) and incubated for 18 h at 37˚C. The gel was stained
with a solution of 0.25% Coomassie brilliant blue R-250
and destained in 7% acetic acid and 10% methanol. The
gelatinolytic regions were observed as white bands against a
blue background. The levels of MMP activity were assessed
by measuring the optical density of the bands by a comput-
erized image analysis.

**In vivo anti-tumor activity.** The tumor mass of colon
carcinoma 26 (C26) passaged in BALB/c mice was excised
and homogenized with saline at a ratio of 1 to 3. Then 0.2 ml
cell suspension was inoculated subcutaneously into each
experimental animal on day 0. Treatment was started on
day 1. Mice were divided into groups and administered i.g. (intra
gastric gavage) with saline (as control) and SPFX
respectively, twice a day for a total of 10 days. All animals
were sacrificed on day 14 and the subcutaneous tumors
were excised and weighed.

Athymic female BALB/c (nu/nu) mice (20±2 g, obtained
from the Institute for Experimental Animals, Chinese Academy
of Medical Science, China) at the age of 4-6 weeks were
used for HCT116 human colon tumor xenografts. HCT116
tumor mass for implantation were initially grown subcu-
aneously by inoculation of HCT116 cells at a dose of 5x10⁶
cells/mouse. A tumor piece of 2-3 mm in diameter was
implanted subcutaneously into each nude mouse. Treatment
was initiated when the tumor volume reached about 100 mm³.
The mice were randomly divided into groups. SPFX was oral
administered twice a day for 20 days. 5-FU was injected
every 3 days intraperitoneally. Tumor growth was followed
every other day by measuring tumor length and width using a
caliper. Tumor volume was calculated using the formula:
Length x width²/2. At the end of the experiment, the mice
were sacrificed and tumor weight was measured.

All the experiments were performed with the protocols
approved by the Animal Care and Use Committee, Institute
of Medicinal Biotechnology, Chinese Academy of Medical
Sciences.

**Statistical analysis.** The data are the mean values of at least
3 experiments and are expressed as mean ± SD. The Student's
t-test was used to compare data. p<0.05 was considered to
be statistically significant.

**Results**

**HERG expression and growth-inhibitory effect of SPFX on the colon carcinoma cell lines.** The expression levels of
HERG protein in cell lines were determined by Western blot
analysis. As shown in Fig.1A, the HERG protein was
expressed in the tested human colon carcinoma cell lines
HCT116 and HT-29, and the MERG protein was expressed
in mouse colon carcinoma cell line C26. The detected
molecular weights with the antibody were about 155-kDa
(HERG) and 205-kDa (MERG) respectively, consistent with
other study (27).

The MTT assay was used to evaluate the inhibitory effect
of SPFX on cell viability. The three cell lines were treated
with SPFX of different concentrations for 48 h. All of
them showed decreased cell viability (Fig. 1A). The HCT116
cells were most sensitive to SPFX, and the IC₅₀ value is
74.83±7.46 μmol/l. The IC₅₀ values in HT-29 and C26 cells
were 137.57±15.27 and 110.23±13.57 μmol/l, respectively.

Herg-transfected HEK293 cells were employed to investi-
gate the relationship of cell viability of SPFX on cancer
cells and HERG expression. In Fig. 1B, the expression of
HERG protein in herg-transfected HEK293 cells (293/herg)
was remarkably increased while no HERG protein expressed
in wild-type HEK293 cells and mock-transfected HEK293
cells (293/mock). The IC₅₀ values of wild-type HEK293 cells
was 145.56 μmol/l. When it was transfected, the pcDNA3.1/
herg IC₅₀ value was evidently declined to 57.65 μmol/l. The
IC₅₀ value in HEK293/herg cells was lower than that in the
wild-type HEK293 cells or HEK293/mock cells
(p<0.05).

Further experiments were performed to inhibit HERG
expression by transfection with HERG-specific small inter-
fering RNA (siRNA). The silencing effect of shRNA-HERG
on the expression of HERG protein in HCT116 cells was
detected (Fig. 1C). The IC₅₀ values of wild-type HCT116
cells, shRNA-control HCT116 cells and shRNA-herg HCT116
cells were 62.53±6.25, 52.83±7.34 and 106.36±12.43 μmol/l,
respectively. The IC₅₀ value in shRNA-herg HCT116 cells
was higher than that in the wild-type HCT116 cells and
shRNA-control HCT116 cells (p<0.05).

**Apoptosis induction by SPFX in colon carcinoma cell lines.**
To further examine the mechanism of inhibiting cell viability
by SPFX, HCT116 cells were treated with different concen-
trations of SPFX and examined for apoptotic cells by PI
single staining and Annexin V-FITC/PI double staining.
After 48 h of exposure to the SPFX, the sub-G1 percentage
of cells increased. SPFX induced apoptosis of HCT116 cells
in a dose-dependent manner (Fig. 2A). The apoptotic cells induced by 100, 200 and 300 μmol/l SPFX reached to 18.07±2.06 (p<0.001), 20.33±3.72 (p<0.001) and 27.40±3.21% (p<0.001), respectively.

The same tendency of inducing apoptosis was confirmed by flow cytometry analysis with FITC-Annexin V and PI double staining (Fig. 2B). The ratio of apoptosis by the double staining was much higher than PI single staining. As Fig. 2C demonstrated, the apoptotic cells induced by 100, 200 and 300 μmol/l SPFX reached 18.62±2.57 (p<0.001), 40.76±2.78 (p<0.001) and 49.55±3.01% (p<0.001), respectively.

Western blot analysis was used to detect the expression of procaspase-3 and Bcl-2 correlation with apoptotic cell death in HCT116 and HT-29 cells. SPFX led to a dose-dependent decrease of the expression of procaspase-3 and Bcl-2 in HCT116 and HT-29 cells (Fig. 3).

The difference of apoptotic rate in wild-type HEK293 cells and herg-transfected HEK293 cells induced with SPFX was also detected by flow cytometry analysis with FITC-
Annexin V and PI double staining. In the wild-type HEK293 cells, less cells appeared apoptotic; however, in 293/herg cells, higher concentrations of SPFX induced eminent apoptosis (Fig. 4A). Meanwhile, compared with wild-type HCT116 cells, the shRNA-herg HCT116 cells that are HERG protein knocked down showed few apoptosis at exposure to SPFX (Fig. 4B).

Decrease of motility and invasion by SPFX in HCT116 cells.

We examined whether SPFX decreases the motility and invasion of HCT116 cells. Figure 2A shows the percentage of sub-G1 cells, which are indicative of apoptosis, after treatment with different concentrations of SPFX. The results indicate that SPFX induces apoptosis in HCT116 cells in a concentration-dependent manner. Figure 2B and 2C show the flow cytometric histograms and bar graphs, respectively, of Annexin V and PI double staining. The data are presented as mean ± SD of three independent experiments. "p<0.001 vs. control.

Figure 2. Apoptosis induction by SPFX in HCT116 cells. (A) Apoptosis was measured by propidium iodide using a flow cytometry analysis. Data represent the means ± SD of three independent experiments. *p<0.001 vs. control. (B) Flow cytometric histograms of HCT116 cells after treated by different concentrations of SPFX for 48 h. Cells were harvested and labeled with a combination of Annexin-FITC and propidium iodide. (C) Apoptosis induction by SPFX in HCT116 cells detected by FITC-Annexin V/PI. Cells treated with different concentrations of SPFX for 48 h. Double staining with FITC-Annexin V and PI for flow cytometry analysis was performed. Data represent the means ± SD of three independent experiments. *p<0.001 vs. control.

Figure 3. Effects of SPFX on expression of apoptosis related proteins in HCT116 and HT-29 cell lines. Cells were incubated with the indicated concentrations of SPFX for 48 h and proteins were analyzed in the cell lysates by Western blot analysis. Blots were stripped and reprobed for Actin as a loading and transfer control.

Figure 4. Apoptosis induction by SPFX in herg-transfected HEK293 cells and herg-knockdown HCT116 cells. Cells were exposed to different concentrations of SPFX for 48 h after 8 h transfection. Double staining with FITC-Annexin V and PI for flow cytometry analysis was performed. Cell apoptosis was determined by FITC-Annexin V and PI. Results were derived from three independent experiments. The data are presented as mean ± SD. *p<0.05, **p<0.01, between indicated groups using a Student's t-test.
the invasion of HCT116 cells. In a wound closure assay, migration of HCT116 cells was inhibited by addition of various concentrations of SPFX for 36 h (Fig. 5A). Next, cell motility was examined using a non-coated chamber assay and cell migration was examined using a chamber coated with matrigel, which mimics the extracellular matrix treated with different concentrations of SPFX for 24 h. As shown in Fig. 5B, SPFX reduced the motility and invasion of HCT116 cells in a dose-dependent manner.

As determined by gelatin zymography assay, the secretion of MMP-2 and MMP-9 from HCT116 cells was markedly decreased after 48-h exposure to SPFX; especially treatment at the concentrations of 300 μmol/l dramatically reduced the MMP-9 and MMP-2. These findings suggest that SPFX inhibits the invasion of cells possibly by inhibiting the secretion of MMP (Fig. 5C).

**Synergism of SPFX in combination with 5-FU inhibited the proliferation in HCT116 and HT-29 cells.** In the present study the effects of 5-FU combined with SPFX were examined in HCT116 cells. In MTT assay, the cells were incubated with various concentrations of 5-FU (2-16 μmol/l) and SPFX 10 μmol/l for 72 h. Addition of 10 μmol/l SPFX alone, a drug concentration that can achieve about 20% inhibitory rate in HCT116 cells. However, when added simultaneously with 5-FU, the cytotoxicity of combined drugs markedly enhanced. The CDI is shown in Fig. 6A (left). Strong synergistic effects were achieved at this dose. The CDI was <0.70 at the combination dose of 10 μmol/l SPFX plus 5-FU. Combination effects were also observed in HT-29 cells. Thirty μmol/l SPFX also provided synergistic cytotoxicity with 5-FU in HT-29 cells in MTT assay. As Fig. 6A (right) illustrates, slight to moderate synergistic growth inhibitory effects (CDI <1) at majority of combinations were found in HT-29 cells.

**SPFX combined with 5-FU enhanced induction of apoptosis in HCT116 cells.** Flow cytometry combined with FITC-Annexin V/PI analysis showed that the combination treatment enhanced the induction of apoptosis in HCT116 cells. As shown in Fig. 6B, 5 μmol/l 5-FU plus 100 and 200 μmol/l SPFX induced apoptosis in 47.59±2.64 and 53.76±5.49%, whereas the same doses of 5 μmol/l 5-FU and SPFX given alone resulted in apoptosis in only 7.24±2.09,
23.90±2.12 and 45.02±4.21%, respectively. Flow cytometry combined with PI single stain analysis showed similar effects. As shown in Fig. 6C, 5 μmol/l 5-FU plus 100 and 200 μmol/l SPFX induced apoptosis in 23.99±5.64 and 32.76±6.49%, whereas the same doses of 5 μmol/l 5-FU and SPFX given alone resulted in apoptosis in only 2.57±0.59, 16.10±3.8 and 17.86±4.39% of control, respectively.

Effect of SPFX on growth of murine carcinoma C26 and human colon carcinoma HCT116 xenografts. BALB/c mice bearing subcutaneous C26 carcinoma were orally administered with PBS, 800 and 1200 mg/kg SPFX. SPFX at the dose of 800 and 1200 mg/kg inhibited the growth of C26 by 36.2 and 69.1%, respectively (Fig. 7A). The mice did not lose body weight significantly and did not show any gross abnormalities upon necropsy at the end of the treatment, which indicated the mice tolerated well these given doses.

The effects of the combination of SPFX and 5-FU on human colon carcinoma HCT116 xenografts were determined. The lower dose of 5-FU (20 mg/kg, every 3 days) was used. Combinatory treatment of SPFX plus 5-FU was well tolerated as shown by maintenance of body weight (Fig. 7B) and by the absence of other signs of toxicity. The growth of the established s.c. tumors in the nude mice was decreased significantly when SPFX was given over a period of 20 days compared with the control saline-treated animals. The resulting growth curves for HCT116 xenografts by various treatment schedules are shown in Fig. 7C. SPFX alone (800 mg/kg) or in combination with 5-FU showed a tumor growth inhibitory rate of 44.2 and 62.4% on day 32, respectively (Table I). SPFX in combination with 5-FU showed stronger inhibitory effect than SPFX or 5-FU alone on tumor growth.

Discussion

HERG can be a specific tumor marker in gastric and colorectal tissues (28,29). Some reports also indicated that several drugs could block HERG potassium as an adjuvant cancer therapy (10,17,30).

In the present study, we found that SPFX could lead to the loss of viable cells. SPFX is difficult to dissolve in water, slightly soluble in DMSO and soluble in alkaline solution. The largest drug solubility of SPFX in 0.1 N NaOH was prepared as a 50 mmol/l stock solution. In MTT assays, the maximum dose which HCT116 cells could tolerate was 0.5 mmol/l. We compared the solvent effect on cell viability.
There was no statistically significant difference between the cell number treated with 500 μmol/l SPFX and those treated with NaOH (in the same concentration) alone (neutral pH value). The range of IC50 values was 74.83-137.57 μmol/l in the colon cancer cells with HERG-overexpression. It was observed that the chemosensitivity of cells to SPFX could be correlated with HERG expression by transient transfection of pCDNA3.1/herg plasmid in HEK293 cells. HEK293 cells were chosen because they have no expression of HERG protein. Chemosensitivity to SPFX in the herg gene transfected HEK293 cells was increased compared to the wild-type or mock HEK293 cells. Similar result of the relationship between the herg expression and chemosensitivity to SPFX was observed in herg-downregulated HCT116 cell by siRNA. RNA interference is a gene silencing technique with high efficiency and specificity. The constructed shRNA expression plasmid could silence herg gene effectively in SH-SY5Y cell growth in vitro and in vivo (31). In this study, after inducing herg-downregulation by transfecting special siRNA plasmid, the chemosensitivity to SPFX in herg-downregulated HCT116 cells was decreased. By comparison of the chemosensitivity conferred by the existence or depletion of HERG protein, it is suggested that SPFX-induced inhibition of survival in cancer cells was, at least in part, related to the inhibition of HERG channel.

The ability of SPFX to induce apoptosis was investigated in cancer cells. The results are consistent with cell growth inhibition studies by MTT, suggesting that the loss of viable cells by SPFX is partly due to the induction of an apoptotic cell death mechanism. The present experiments on both herg gene transfected HEK293 cells and herg-downregulated HCT116 cells confirmed that apoptosis induced by SPFX was also related with HERG expression. However, the precise molecular mechanisms on SPFX inducing apoptotic cell death need to be elucidated. Classical apoptosis pathways are Fas/FasL mediated and Bcl-2 mediated mitochondria pathways, of which caspase-3 is the key enzyme. The anti-apoptotic properties of Bcl-2 have been attributed to their ability to prevent translocation of cytochrome c to the cytosol, and thus, interfere with the subsequent activation of cytosolic caspases and apoptosis (32,33). In the present

Table I. Inhibitory effects of SPFX combined with 5-FU on the growth of human colon carcinoma HCT116 xenografts in nude mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>No. of mice</th>
<th>Initial</th>
<th>End</th>
<th>BWC (g)</th>
<th>Tumor weight (g)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>10</td>
<td>Control</td>
<td>6</td>
<td>6</td>
<td>-1.87</td>
<td>1.81±0.33</td>
<td>24.9</td>
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<tr>
<td>5-FU</td>
<td>20</td>
<td>5-FU</td>
<td>6</td>
<td>6</td>
<td>0.22</td>
<td>1.36±0.50</td>
<td>44.2</td>
</tr>
<tr>
<td>SPFX</td>
<td>800</td>
<td>SPFX</td>
<td>6</td>
<td>6</td>
<td>-0.94</td>
<td>1.01±0.36</td>
<td>44.2</td>
</tr>
<tr>
<td>SPFX</td>
<td>1200</td>
<td>SPF+SPFX</td>
<td>6</td>
<td>6</td>
<td>-1.23</td>
<td>0.76±0.13</td>
<td>58.0</td>
</tr>
<tr>
<td>5-FU+SPFX</td>
<td>20+800</td>
<td>SPF+SPFX</td>
<td>6</td>
<td>6</td>
<td>-0.94</td>
<td>0.68±0.34</td>
<td>62.4</td>
</tr>
</tbody>
</table>

Results were recorded on day 32 after tumor implantation; SPFX, sparflloxacin; 5-FU, 5-fluorouracil; 5-FU was injected intraperitoneally daily and SPFX was orally administered twice a day for 20 days; BWC (g), average body weight change; CDI, 0.9, *p<0.05, p<0.01 vs. the control group.
study, we found that SPFX induced cell killing by reducing the expression of procaspase-3 and Bcl-2 proteins.

Degradation of the extracellular matrix in tissue surrounding the tumor is a critical event in the process of cancer invasion and metastasis. The first barrier for an invading epithelial tumor is the basement membrane, primarily composed of type IV collagen. MMPs that degrade type IV collagen are increased in colorectal cancer (34) and HERG protein regulates cell invasion of colon cancer cells (14). Through analyzing the inhibitory effect of activity of MMP by SPFX with gelatin zymography, we concluded that the inhibition of invasive behavior of hrg-expressing colon cancer by SPFX may be related to the suppression of MMP activity.

The combination effect of SPFX and 5-FU in vitro or in vivo was further investigated. The result showed that the combination effect was synergistic when 5-FU was given at different concentrations while SPFX alone was given at the concentration that achieved 80% inhibition. Further data demonstrated that the induction of apoptosis by 5-FU in HCT116 cells was potentiated by SPFX. These data demonstrated SPFX could potentiate the antitumor activity of 5-FU in highly HERG-expressing colon cancer cells. In animal model, the growth inhibitory effect of SPFX was first examined in murine carcinoma C26. The result indicated that SPFX exerts antitumor efficacy at well-tolerated doses. Notably, SPFX alone could inhibit the tumor growth of colon cancer HCT116 xenografts. A combination of SPFX and 5-FU at lower doses inhibited the growth of colon carcinoma HCT116 xenografts more effectively than administration of SPFX or 5-FU alone.

In summary, sparflaxacin, a fluoroquinolone antibacterial agent that has been used for treatment of bacterial infections, can inhibit the proliferation, apoptosis and migration of cancer cells. The combination of sparflaxacin and 5-FU, one of the conventional agents used in the treatment of colon cancer, demonstrated synergistic activity in vitro or in vivo. Sparflaxacin as a modulating agent might be interesting in combination with anticancer drugs for future preclinical and clinical studies.

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