Repertaxin, an inhibitor of the chemokine receptors CXCR1 and CXCR2, inhibits malignant behavior of human gastric cancer MKN45 cells *in vitro* and *in vivo* and enhances efficacy of 5-fluorouracil

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Abstract. Chemokine-mediated activation of G protein-coupled receptors CXCR1/2 promotes tumor growth, invasion, inflammation and metastasis. Repertaxin, a CXCR1/2 small-molecule inhibitor, has been shown to attenuate many of these tumor-associated processes. The present study aimed to investigate the effects of repertaxin alone and in combination with 5-fluorouracil (5-FU) on the malignant behavior of gastric cancer and the potential mechanisms. Gastric cancer MKN45 cells were treated *in vitro* with repertaxin and 5-FU, either alone or in combination. MTT and colony formation assay were performed to assess proliferation. Cell cycle progression and apoptosis was completed by flow cytometry. Migration and invasion were also assessed by Transwell and wound-healing assay. Western blot analysis and quantitative RT-PCR were performed to determine expression of signaling molecules. MKN45 cells were also grown as xenografts in nude mice. Mice were treated with repertaxin and 5-FU, and tumor volume and weight, angiogenesis, proliferation and apoptosis were monitored. Combination of repertaxin and 5-FU inhibited MKN45 cell proliferation and increased apoptosis better than either agent alone. Similarly, enhanced effect of the combination was also observed in migration and invasion assays. The improved effect of repertaxin and 5-FU was also observed *in vivo*, as xenograft models treated with both compounds exhibited significantly decreased tumor volume and increased apoptosis. In conclusion, repertaxin inhibited malignant behavior of human gastric cancer MKN45 cells *in vitro* and *in vivo* and enhances efficacy of 5-fluorouracil. These data provide rationale that targeting CXCR1/2 with small molecule inhibitors may enhance chemotherapeutic efficacy for the treatment of gastric cancer.

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Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide (1,2), with a particularly high rate in China (3). Despite recent advances in screening and treatment, the 5-year survival rate of advanced gastric adenocarcinoma patients is still low (4). Therefore, improved therapies are essential. To date, many gastric cancer patients are treated with chemotherapy, such as 5-fluorouracil (5-FU). Uncontrolled proliferation, invasion and metastasis are the major poor prognostic factors associated with advanced gastric cancer (5). Additionally, there is a close relationship between inflammation and cancer. This relationship is not new. It was originally described in 1863 by Virchow, who hypothesized that cancer originates at sites of chronic inflammation (6). Non-resolving inflammation has been linked to the development and progression of gastric cancer (7,8). In many cases, invading inflammatory cells can secrete growth factors or cytokines that act directly on tumor cells or on the stroma to promote cellular proliferation. Moreover, chemokine receptors and their ligands have been linked to inflammation and have been associated with cancer progression (9-12). Some cancer cells express high levels of chemokine receptors, such as CXCR1/2 (13-15). The interaction between chemokine receptors and their ligands has been shown to regulate multiple aspects of cancer cell biology, including interaction with the tumor microenvironment (16,17), cell survival, growth (18), angiogenesis (16), invasion (19) and metastasis (11,12,20).

CXCR1/2 are G protein-coupled receptors that bind interleukin-8 (IL-8) and transduce its signal via a G-protein-activating second messenger system. CXCR1 and CXCR2 share 76% amino acid identity, with the highest homology

occurring within the transmembrane domains (21). CXCR1/2 are mainly expressed on neutrophils and were originally characterized by their ability to induce chemotaxis of leukocytes, but recent study has demonstrated that these receptors may play a critical role in certain cancers. CXCR1/2 are overexpressed in numerous solid tumors and in several cancer cell lines, including the gastric cancer cell line MKN45 (22,23). Several studies have correlated expression of CXCR1/2 with increased proliferation, invasion, metastasis and resistance to therapy (24-28). Many of these cancer phenotypes are mediated by the binding of IL-8 to these receptors. In fact, most primary and metastatic tumors constitutively express IL-8. Thus, chemokine signaling can promote tumorigenesis and may represent a useful therapeutic target in cancer.

Despite recent advances (22,29), the exact role of CXCR1/2 in gastric cancer remains unclear. However, several studies provide evidence that inhibition of CXCR1/2 signaling may be a viable strategy for cancer treatment showing that inhibition of these receptors can slow tumor progression (30-32). Repertaxin is a non-competitive allosteric inhibitor of CXCR1/2. Upon binding, repertaxin locks these receptors in an inactive conformation, which prevents receptor signaling (33). Although repertaxin was originally developed to inhibit CXCR1/2 function to reduce reperfusion injury (34,35), the importance of chemokine signaling in cancer raises the possibility that repertaxin may also be useful as an anticancer agent. In fact, clinical phase I studies using this compound to treat reperfusion injury have demonstrated a lack of toxicity (26). This finding significantly increases the potential utility of repertaxin as an anticancer therapy (26). While some reports have begun to explore this (36,37), the potential efficacy of repertaxin in gastric cancer has not been examined.

In the present study, we used both gastric cancer cell lines and mouse xenograft models to investigate the effects of the CXCR1/2 inhibitor repertaxin, either alone or in combination with the commonly administered gastric cancer chemotherapy 5-FU. We assessed the effect of repertaxin and 5-FU on several tumorigenic phenotypes, including proliferation, cell cycle, apoptosis and migration and invasion. We found that combined administration of repertaxin and 5-FU significantly attenuates many of these phenotypes when compared to treatment with either compound alone, suggesting that combined inhibition may be clinically useful for the treatment of gastric cancer.

Materials and methods

Cell culture. The poorly differentiated gastric carcinoma cell line MKN45 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; (Gibco-BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified cell culture incubator at 37˚C and in 5% CO_2.

Detection of IL-8 by ELISA. The level of soluble IL-8 secreted into the culture medium of MKN45 cells was performed using enzyme linked-immunosorbent assay (ELISA; Sigma-Aldrich, St. Louis, CA, USA) according to the manufacturer's instructions. In brief, related reagents and samples were prepared, and the samples containing IL-8 were added to each well at 100 μl. After incubation for 90 min, plates were incubated with a biotinylated antibody. Immunoreactivity was determined using the avidin-HRP-TMB detection system. The reactions were stopped by addition of TMB stop buffer, and absorbance was measured at 450 nm using a microplate reader (Bio-Rad 680). A curve of the absorbance vs. concentrations of standard wells was plotted. The concentration of human IL-8 in the tested samples was determined by comparing the absorbance of the samples to the standard curve.

Cell viability assay. Cells were seeded in a 96-well plate at a density of 5,000 cells/well for MTT assays. Treatments with different concentrations of repertaxin (dissolved in 0.9% saline) alone (0.025, 0.25, 2.5, 25 and 50 μg/ml) (Sigma-Aldrich), 5-FU alone (0.01, 0.1, 1, 10 and 20 μg/ml) (Shanghai Xudong Haipu Pharmaceutical, Co., Ltd., Shanghai, China), and repertaxin (0.025, 0.25, 2.5, 25 and 50 μg/ml) in combination with 5-FU (0.01, 0.1, 1, 10 and 20 μg/ml) were administered 12 h after seeding. Cell viability was determined on days 1, 2, 3, 4 and 5 after treatment by addition of 20 μl MTT solution (5 mg/ml in PBS) (Sigma-Aldrich). Cells were incubated in MTT solution for 4 h at 37˚C. Next, the culture medium was removed and replaced with 150 μl DMSO to dissolve the crystals. Samples were incubated at room temperature for 10 min, and then absorbance was measured at 568 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Colony forming assay. Cells were seeded in a 6-well plate at a density of 200 cells/well and cultured for two weeks. After the medium was removed, cells were washed three times with PBS and fixed for 15 min in pure methanol, and then stained for 25 min with crystal violet (0.5% w/v) (Sigma). Colonies of more than 50 cells were identified using an inverted microscope (Olympus IX70; Olympus Corp., Tokyo, Japan). Colony efficiency (CE) and the rate of colony inhibition (CI) were calculated as follow: CE = (colonies formed/cells seeded) x 100% and CI = (CE in experimental group/CE in control group) x 100%, respectively.

Cell cycle analysis. MKN45 cells were plated in 60-mm dishes and grown to 50% confluence. Complete medium was then removed and replaced with medium containing repertaxin and/or 5-FU. After 48 h, treated cells were harvested and fixed overnight with cold 70% ethanol at -20˚C. After washing with PBS, the samples were incubated with 50 μg/ml propidium iodide (PI; Sigma-Aldrich), 100 μg/ml RNase A and 0.2% Triton X-100 in the dark for 30 min. Flow cytometric analysis was performed using flow cytometry system and ModFit software (B&D Biosciences, San Diego, CA, USA) (38). Cell cycle distribution, including the percentage of cells in G0/G1, S and G2/M was obtained.

Apoptosis assay. Cells were treated with repertaxin and/or 5-FU and then incubated in buffer containing Annexin V-fluorescein isothiocyanate (0.5 μg/ml; Annexin V-FITC apoptosis detection kit; Sigma-Aldrich) and PI (0.6 μg/ml) in the dark, at room temperature for 15 min. Samples were then measured by flow

Table I. Primer sequences used for quantitative real-time RT-PCR.

Gene	Forward primer	Reverse primer	Product length (bp)
GAPDH	5'-TGAACGGGAAGCTCACTGG-3'	5'-TCCACCACCCTGTTGCTGTA-3'	307
Bax	5'-CCCGAGAGGTCTTTTTCCGAG-3'	5'-CCAGCCCATGATGGTTCTGAT-3'	155
Bcl-2	5'-CCTGGGCAATTCCGCATT-3'	5'-AACAGGCCACGTAAAGCAAC-3'	158
Cyclin D1	5'-GCTGCGAAGTGGAAACCATC-3'	5'-CCTCCTTCTGCACACATTTGAA-3'	135
EGFR	5'-AGGCACGAGTAACAAGCTCAC-3'	5'-ATGAGGACATAACCAGCCACC-3'	177
VEGF	5'-ATTATGCGGATCAAACCTC-3'	5'-ATTTCTTGCGCTTTCGTT-3'	157
MMP-9	5'-ACTACTGTGCCTTTGAGTCC-3'	5'-AGAATCGCCAGTACTTCCCA-3'	115
MMP-2	5'-ACTCTGGACTTAGACCGCTTG-3'	5'-ACAGGTTGCAGCTCTCCTTG-3'	217
TIMP-2	5'-ACCCCTGTTCGCTTCCTGT-3'	5'-GGGTCAAATGCTTCCACGAT-3'	196
E-cadherin	5'-GCTAACGTCGTAATCACCAC-3'	5'-AATGCCATCGTTGTTCACTG-3'	141
Ki-67	5'-AGAAGACCTGCTACTCCAAAGA-3'	5'-AGTTTGCGTGGCCTGTACTAA-3'	70

cytometry (B&D Biosciences), and the percentage of apoptotic cells was calculated using CellQuest software.

Cell migration and invasion assay. Cell migration and invasion were performed using Transwell inserts (Corning Costar) with polycarbonate membranes (8 μm pore size) in 24-well plates. For invasion assays, the filter was coated with 20 μg/ml Matrigel (Becton-Dickinson) overnight at 4˚C. A total of 100 μl cells at 1.0x10^5/ml in serum-free medium containing the indicated compounds, were seeded in the upper chamber. The lower chamber was also treated with compounds in 800 μl culture medium containing 10% FBS. After 24-h incubation at 37˚C in 5% CO_2, non-invading cells were removed from the upper surface of the membrane by gently scrubbing with a cotton swab. Cells that had successfully invaded the lower surface of the membrane were fixed with methanol, stained with crystal violet, rinsed with water and then air dried. The invading cells were photographed and counted using an inverted microscope in ten random fields (magnification, x200). Experimental conditions for migration assays were similar to those used for invasion assays with the exception of Matrigel-coated chambers.

Wound-healing assay. Wound-healing assays were performed to assess cancer cell migration. Briefly, MKN45 cells were seeded at a density of 10^5 cells/well in a 6-well plate and grown to near confluent monolayers in DMEM containing 10% FBS. Wounds were made by scratching the cell monolayer with a sterile 200 μl pipette tip. The cells were then washed twice with PBS. The scratched areas were photographed at x10 magnification using computer-assisted microscopy (Olympus IX70; Olympus Corp.). Images were captured at 0, 24, 48 and 72 h after the scratch was made. Images were analyzed using CellProfiler 2.0 cell image analysis software (http://www.cellprofiler.org) (39).

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from cells was isolated using TRIzol reagent (Invitrogen) and quantified spectrophotometrically. Single-stranded cDNA was synthesized using SuperScript First-Strand Synthesis System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Sequences of all primers used are listed in Table I. All real-time PCR reactions were performed with SYBR-Green Master Mix kit (all-in-One™ qPCR SYBR-Green I Mix from GeneCopoeia, Rockville, MD, USA) using an ABI PRISM 7500 sequence detection system. GADPH was used as an internal loading control. The reactions were performed as follows: denaturation for 10 min at 95˚C, 40 cycles of 95˚C for 10 sec, and 60˚C for 20 sec. The $2^{-\Delta\Delta Ct}$ method was performed to calculate the relative mRNA expression of target genes.

Western blot analysis. Cells were lysed in buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100) containing protease inhibitor, and then samples were centrifuged at 12,000 rpm for 12 min at 4˚C. Equal amounts of protein (50 μg), quantified by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA), were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8-12%). After electrophoresis, separated proteins were transferred from the gel to polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were then incubated in blocking solution containing 5% non-fat milk for 1.5 h. After blocking, membranes were incubated with primary antibody. Phosphorylated antibodies (p-AKT-Ser^{473}, anti-AKT, p-ERK-Thr^{202}/Tyr^{204} and anti-ERK1/2) were obtained from Anbo Biotechnology Co., Ltd. (San Francisco, CA, USA). Additionally, primary antibodies detecting CXCR1, CXCR2, Bcl-2, Bax, cyclin D1, EGFR, Ki-67, VEGF, MMP-9, MMP-2, TIMP-2 and E-cadherin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); each of these antibodies were prepared at a 1:500 dilution in TBST overnight at 4˚C. Following three washes, the blots were incubated with the appropriate horseradish peroxidase conjugated secondary antibody (1:2,000 dilution in TBST) for 1 h at room temperature. GADPH was used as a loading control. Proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology).

MKN45 gastric carcinoma xenograft mouse model. Four to six-week old athymic female Balb/c-nu/nu nude mice with a

body weight of 15-20 g were obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). Mice were housed in individually ventilated cages with isolated ventilation at the Animal Care Center of the Third Xiang-ya Hospital, Central South University under specific pathogen-free conditions. Animals were allowed to adapt to their environment for 1 week prior to experimentation.

Twenty-four mice were randomly divided into four groups, and 0.2 ml $1.0x10^7$ MKN45 cells were implanted subcutaneously (s.c.) into the right back side of each mouse. Once the tumor reached ~5 mm in size (14 days after cell inoculation), we initiated treatment with repertaxin alone (30 mg/kg, s.c. next to the tumor, once every two days for 3 weeks), 5-FU alone (10 mg/kg, s.c. once every two days for 3 weeks), repertaxin plus 5-FU in combination, or saline (s.c. once every two days for 3 weeks), which was used as a control. Tumor volume was measured using digital calipers every other day, and calculated according to the following formula: [length x $(width)^2$]/2. After three weeks of therapy, all mice were euthanized with disoprofol according to the institutional guidelines and local tumors were resected and analyzed. A portion of the tumor tissue was fixed and processed for H&E, immunohistochemistry for PCNA and CD34 and another portion was used for TUNEL staining.

All experimental procedures were conducted in conformity with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Animal Care and Ethics Committee of Third Xiang-ya Hospital, Central South University (no. 2012-10). All procedures were in line with the most recent specifications of the National Research Council (US) Committee for the Care and Use of Laboratory Animals (2011) Guide for the Care and Use of Laboratory Animals (National Academies Press, Washington, DC), 8th edition.

TUNEL. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed to assess apoptosis. Tumor tissue sections were stained according to the manufacturer's instructions (In situ Cell Death Detection kit, fluorescein; Roche Diagnostics, Indianapolis, IN, USA; cat. no. 11684795910). Sections were detected with a fluorescent microscope (Olympus Corp.) and apoptotic cell nuclei were stained in green.

H&E staining and immunohistochemical (IHC) analysis. At least 10 serial, thin (4 μm) sections were cut from each paraffin block. Sections were deparaffinized and then hydrated for hematoxylin and eosin staining and immunohistochemical detection of PCNA and CD34. For immunohistochemistry, endogenous peroxidase activity was inhibited, and then slides were treated with antigen retrieval buffer (citrate buffer, pH 6.0, at 100˚C, 2 min or EDTA buffer, pH 8.0, at 100˚C, 2 min in a pressure cooker). Samples were then blocked in normal goat serum and stained with primary antibodies including PCNA (Santa Cruz Biotechnology) and CD34 (Santa Cruz Biotechnology) at 4˚C overnight. PBS, instead of primary antibody, was applied as a negative control. Slides were washed and then incubated with the appropriate horseradish peroxidase conjugated secondary antibody for 1 h. DAB was used as a chromogen and sections were counterstained with hematoxylin. CD34-positive microvessels were counted under a light microscope (Olympus Corp.) with 10 high magnification fields each group.

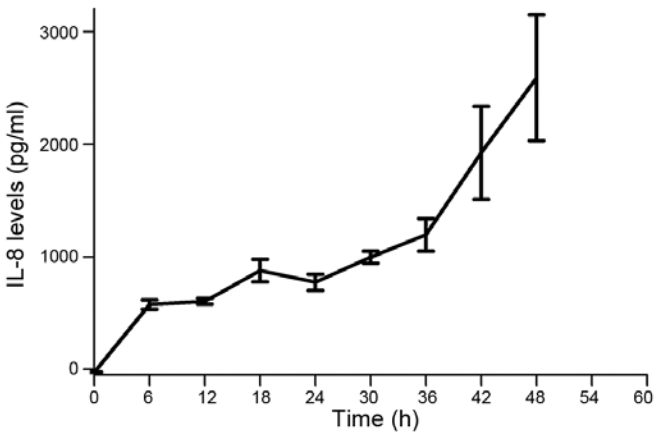

Figure 1. Poorly differentiated gastric carcinoma MKN45 cells secrete IL-8. IL-8 levels in the culture supernatant were determined by ELISA. Data are shown as mean ± standard deviation (SD).

Statistical analysis. The SPSS 13.0 software system (SPSS, Inc., Chicago, IL, USA) was used for statistical evaluation. Data are expressed as mean ± standard deviation (SD) for at least 3 independent experiments. Statistical significance was evaluated by analysis of variance (ANOVA) with Tukey's for post-hoc test. A probability value of <0.05 ($P<0.05$) was considered to indicate a statistically significant result.

Results

MKN45 cells secrete IL-8. Previously it was established that some tumor cells secrete IL-8 as an autocrine growth factor, which can bind CXCR1/2 receptors and promote tumor growth, invasion and metastatic spread (40). We performed ELISA analysis and found that the gastric cancer cell line MKN45 secretes significant levels of IL-8 over time (Fig. 1).

Repertaxin combined with 5-FU inhibits MKN45 cell proliferation and enhances apoptosis. We investigated the effects of repertaxin both alone and in combination with 5-FU on the proliferation of MKN45 gastric cancer cells. Cells were treated with multiple concentrations of repertaxin alone (50, 25, 2.5 0.25 and 0.025 μg/ml) and 5-FU alone (20, 10, 1, 0.1 and 0.01 μg/ml). Both compounds inhibited proliferation in a dose-dependent and time-dependent manner (Fig. 2A and B). Based on these initial experiments, we selected 25 μg/ml repertaxin and 10 μg/ml 5-FU for follow-up experiments.

As shown in Fig. 2C, the anti-proliferative effect of repertaxin in combination with 5-FU was better than that of repertaxin or 5-FU alone. Similar effects were observed in colony formation assays. That is, the combined treatment of repertaxin and 5-FU more significantly ($P<0.05$) inhibited colony formation compared to either compound administered individually (Fig. 2D).

We next examined the effect of repertaxin and 5-FU on cell cycle progression by performing flow cytometric analysis. Compared to control, repertaxin increased the percentage

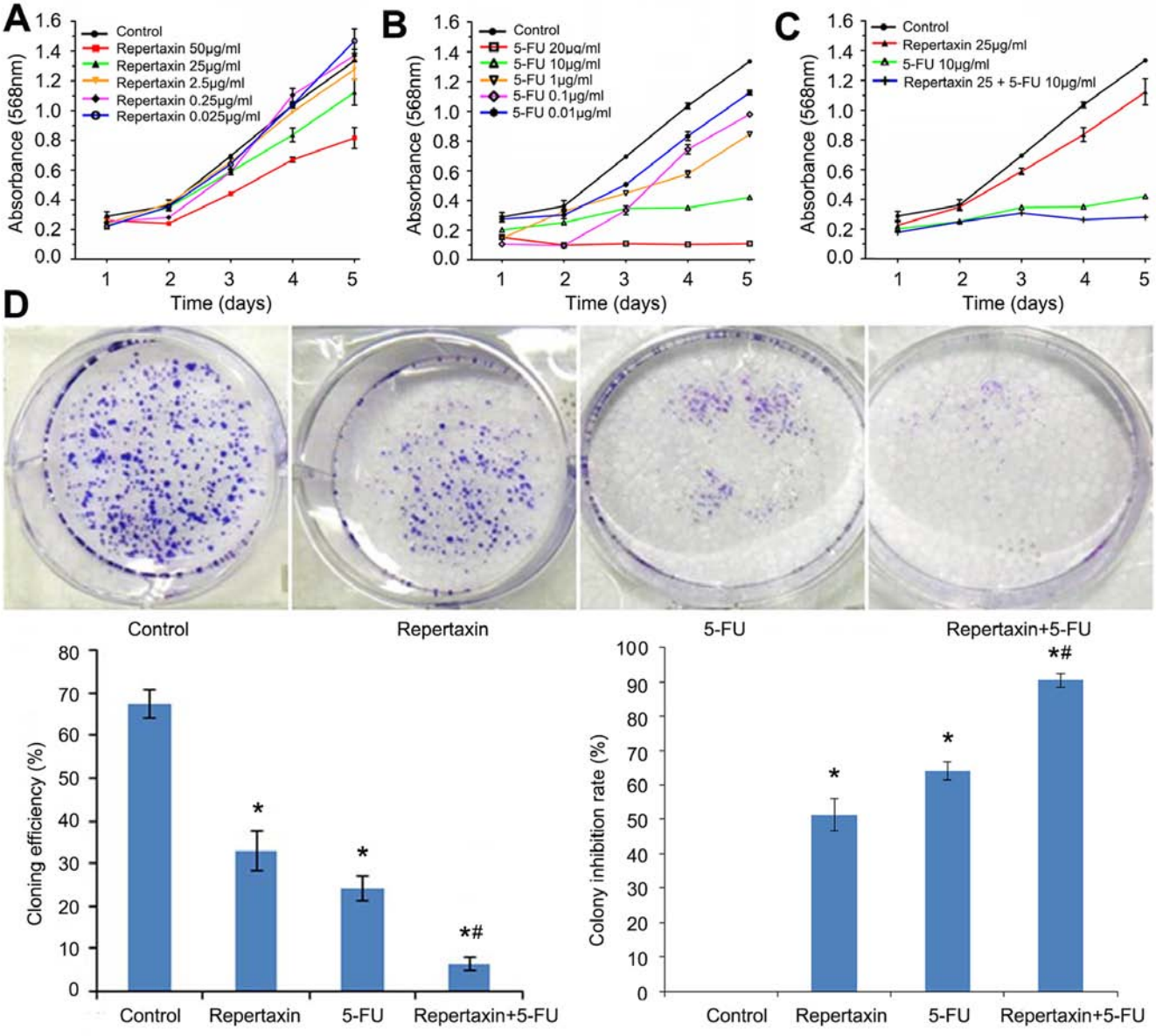

Figure 2. Effects of repertaxin and repertaxin combined with 5-FU on MKN45 cell proliferation and growth. Cell proliferation was analyzed by MTT assay. MKN45 cells were treated with (A) different concentrations of repertaxin alone (0.025, 0.25, 2.5, 25 and 50 μg/ml), (B) 5-FU alone (0.01, 0.1, 1, 10 and 20 μg/ml), and (C) repertaxin (25 μg/ml) in combination with 5-FU (10 μg/ml) for 1, 2, 3, 4 and 5 days. (D) Cell growth was determined by colony forming assay. MKN45 cells were treated with repertaxin (25 μg/ml) and 5-FU (10 μg/ml) alone or in combination. After two weeks, colonies of >50 cells were identified under an inverted microscope. The colony forming efficiency and the colony inhibition rate were calculated. Data are shown as mean ± SD. *P<0.05 vs. control group (0.9% normal saline); #P<0.05 repertaxin+5-FU vs. 5-FU.

of cells in G0/G1 and S phases (96.33 compared to 85.07% in controls) and decreased the percentage of cells in G2/M phase (3.67 compared to 14.93% in controls) (Fig. 3A). These observations were made 24 h after repertaxin (25 μg/ml) treatment. Additionally, the combination of repertaxin and 5-FU performed better than either compound alone (Fig. 3A). We also performed Annexin V/PI staining to assess the effect of repertaxin and 5-FU on apoptosis of MKN45 cells. Repertaxin alone (25 μg/ml) increased the percentage of cells undergoing early (Annexin V^+/PI^-) apoptosis (Fig. 3B). Importantly, the early apoptotic rates of repertaxin alone, 5-FU alone, and repertaxin plus 5-FU were 6.43±1.14, 2.21±0.33 and 9.63±0.78%, respectively. Although all treatment groups were significantly different from controls (1.00±0.32%) (P<0.05), the combination performed significantly better than either agent alone (P<0.05).

Repertaxin combined with 5-FU inhibits MKN45 cell migration and invasion. In addition to effects on cellular proliferation, CXCR1/2 can also influence the migration and invasion of certain tumor cells (41). In the present study, we examined the effect of repertaxin and 5-FU on the migratory and invasive behavior of MKN45 cells. Repertaxin (25 μg/ml) significantly attenuated (P<0.05) chemotaxis migration of MKN45 cells compared to controls (Fig. 4A and C). Similarly, the number of invading cells was also decreased by repertaxin in an *in vitro* Matrigel invasion assay (Fig. 4A and C). Even further inhibition was observed when repertaxin was

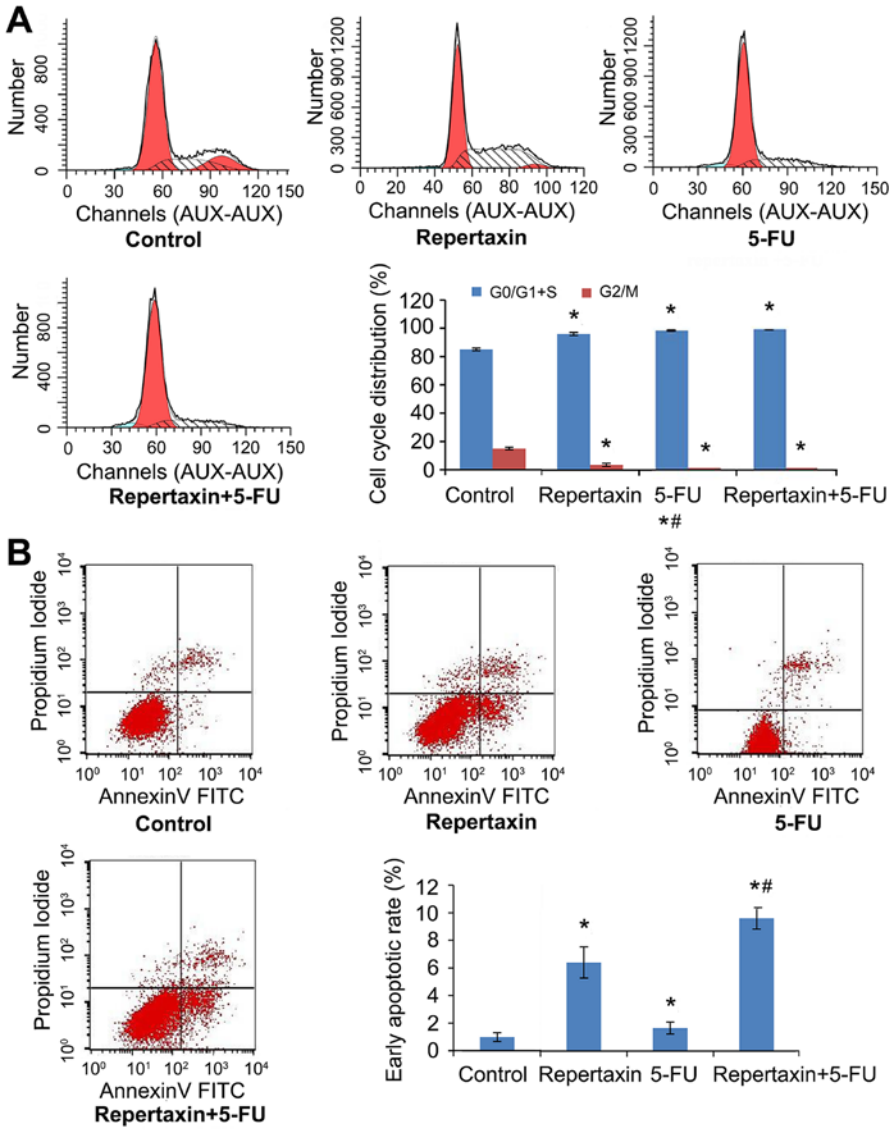

Figure 3. Effects of repertaxin and repertaxin combined with 5-FU on cell cycle distribution and apoptosis in MKN45 cells. MKN45 cells were treated with repertaxin (25 μg/ml) alone, 5-FU (10 μg/ml) alone, or the combination for 24 h. (A) Cell cycle was determined by flow cytometric analysis using PI staining. (B) Early apoptosis was determined by flow cytometric analysis using Annexin V/PI staining. Annexin V was set as the horizontal axis and PI was set as the vertical axis. Mechanically damaged cells are located in the upper left quadrant, apoptotic or necrotic cells in the upper right quadrant, dual negative and normal cells in the lower left quadrant, and early apoptotic cells in the lower right quadrant of the flow cytometric dot plot. Data are shown as mean ± SD. *P<0.05 vs. control group; #P<0.05 repertaxin+5-FU vs. 5-FU.

combined with 5-FU (10 μg/ml) (Fig. 4A and C). In agreement with results from the Transwell assay, data from the wound healing assay also showed significantly improved inhibition of wound closure in the repertaxin-5-FU combination treatment group compared to either the control group or the individual treatments alone (P<0.05) (Fig. 4B and D).

Repertaxin combined with 5-FU significantly reduces gastric cancer cell tumorigenicity and angiogenesis in nude mouse xenografts. To characterize the *in vivo* effects of repertaxin alone and in combination with 5-FU, we established *in vivo* MKN45 xenograft models in nude mice. Mice treated with either repertaxin (30 mg/kg) or 5-FU (10 mg/kg) alone showed significant reduction in tumor volume and weight compared to control-treated mice (Fig. 5A and B). Importantly, combined administration of repertaxin and 5-FU performed better at reducing xenograft tumor growth compared to either agent alone (both P<0.05) (Fig. 5A and B). All treatments were well tolerated, and we did not observe any signs of general toxicity or body weight loss during therapy. Taken together, our findings suggest that combination therapy of repertaxin and 5-FU may cooperate to effectively reduce gastric cancer tumor growth *in vivo*.

This ability of combination treatment to repress tumor growth was followed up with immunohistochemical staining of tumor sections isolated from each of the treatment groups. Thirty-five days following treatment, tumors were excised and subject to molecular analysis. H&E staining showed large number of tumor cells, more heterogeneity and pathological mitosis in the controls, but repertaxin and 5-FU alone or in combination significantly reduced the number of tumor cells, showing less heterogeneity and pathological mitosis (Fig. 5C).

TUNEL staining showed that repertaxin and 5-FU were both individually capable of inducing apoptosis (Fig. 5D);

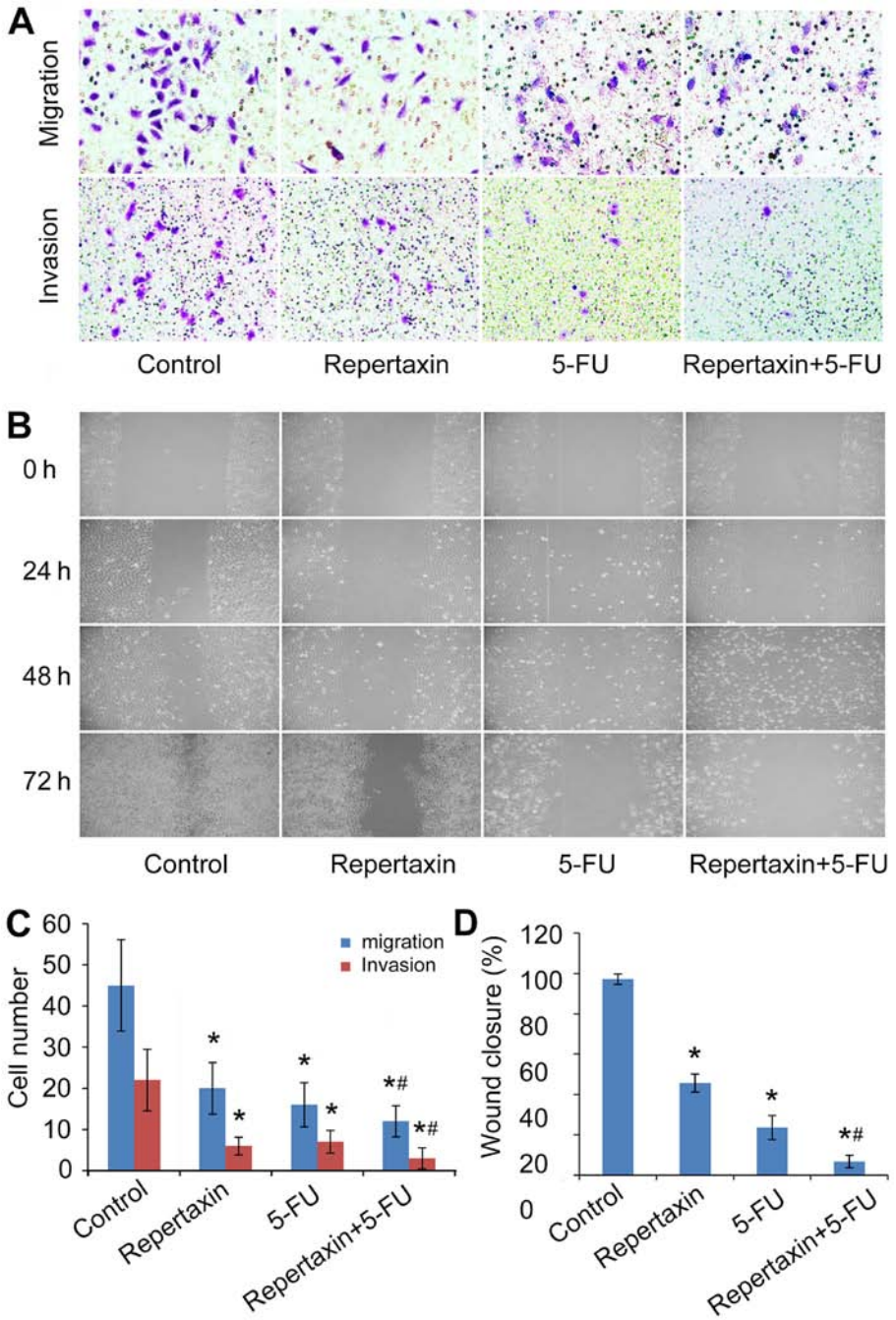

Figure 4. Effects of repertaxin and repertaxin combined with 5-FU on cell migration and invasion in MKN45 cells. MKN45 cells were treated with repertaxin (25 μg/ml) alone, 5-FU (10 μg/ml) alone, or combined repertaxin (25 μg/ml) plus 5-FU (10 μg/ml). (A) MKN45 cells were plated on non-coated or Matrigel-coated membranes for migration (top panel) and invasion (lower panel) assays and incubated for 24 and 48 h, respectively. (B) Wound healing assay: images obtained at 0, 24, 48 and 72 h after scratch formation. (C) Migratory and invasive cells were counted in 10 random fields (x200) and expressed as the average number of cells per field of view. (D) Wound closure (%) = [Cell-free area (0 h) - Cell-free area (72 h)]/Cell-free area (0 h). Data are shown as mean ± SD. *P<0.05 vs. control group; #P<0.05 repertaxin+5-FU vs. 5-FU.

importantly, this effect was further increased when both compounds were administered together.

We next performed PCNA staining to assess the effects on proliferation *in vivo* (42). Repertaxin alone significantly reduced the number of PCNA-positive cells, and combined treatment with 5-FU may have even further decreased the number of proliferating cells (Fig. 5E).

Analysis of apoptosis and proliferation was complemented by examination of angiogenesis, a critical component of gastric cancer growth and metastasis (43,44). Furthermore, the relationship between CXCR1/2 and tumor angiogenesis is well-established (45). The extent of neovascularization of transplanted tumor in nude mice was examined by staining tumor sections with an anti-CD34 antibody and determining microvessel density (MVD) (Fig. 5F). Treatment with repertaxin or 5-FU alone decreased MVD, and the combination of these two compounds may have further decreased the number of MVD/each high-power field (MVD: repertaxin +5-FU: 3.1±1.7; repertaxin: 3.7±1.6; 5-FU: 4.1±1.4 and controls: 6.1±1.9) (Table II).

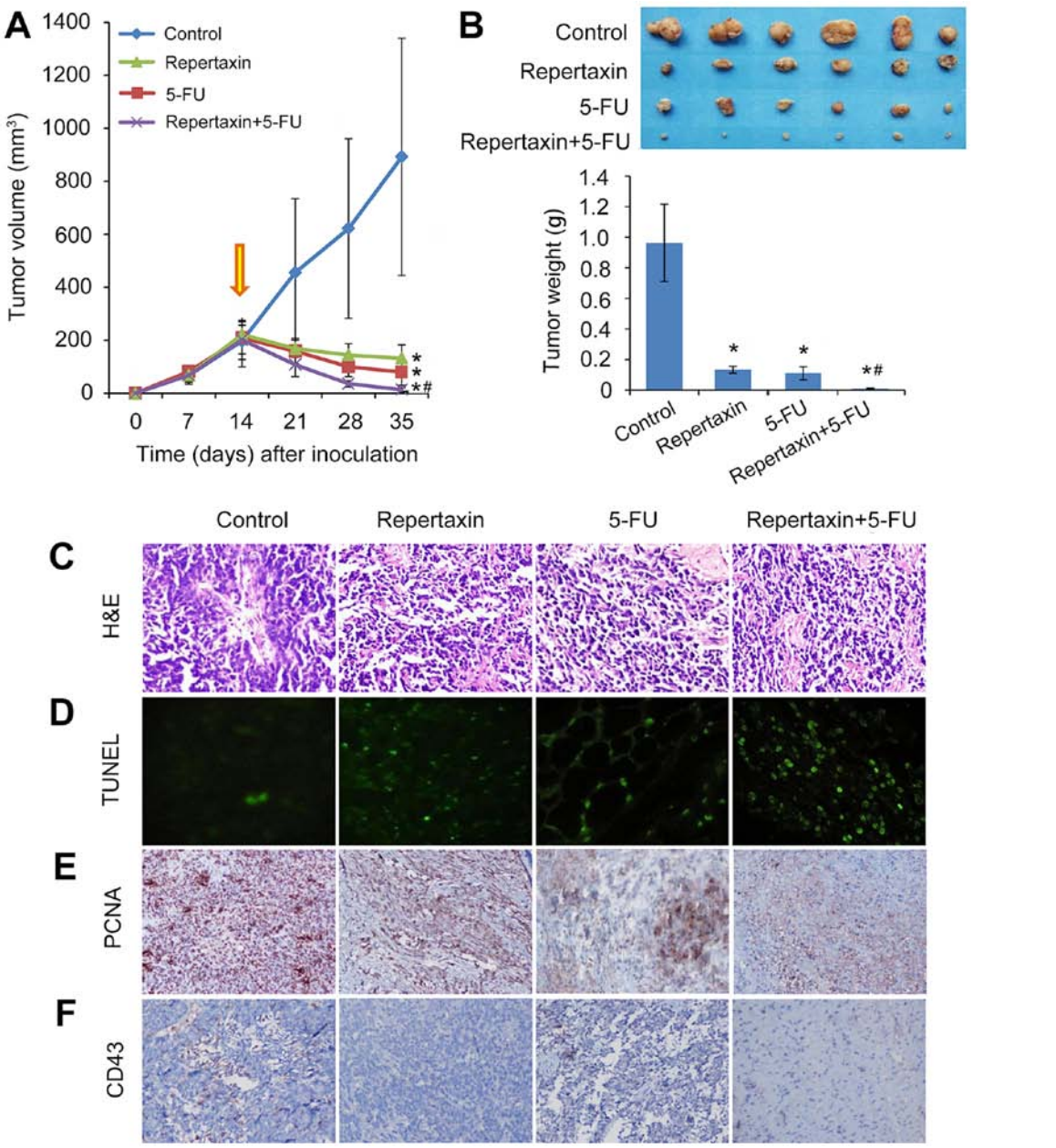

Figure 5. Effects of repertaxin and repertaxin combined with 5-FU on tumor volume, tumor weight and angiogenesis in MKN45 gastric carcinoma xenograft mouse model. Repertaxin alone (30 mg/kg subcutaneously (s.c.) once every two days for 3 weeks), 5-FU alone (10 mg/kg s.c. once every two days for 3 weeks), repertaxin combined with 5-FU (s.c. once every two days for 3 weeks), or a control group injected with saline (s.c. once every two days for 3 weeks). Treatment was initiated on day 14. Nude mice were sacrificed 35 days after inoculation. (A) Tumor volume curves. Treatment was initiated on day 14 (arrow; the tumor size was ~5 mm). (B) Tumor weights. Tumor tissues from each group were collected: hematoxylin and eosin (H&E) (C) TUNEL assay (D) for apoptotic cells and immunohistochemistry staining for PCNA (E) and CD34 (F) (magnification, x200). Apoptotic cell nuclei appear as green fluorescent dots (D). Data are shown as mean ± SD (n=6/group). *P<0.05 vs. control group; #P<0.05 combined treatment group vs. 5-FU alone group.

Table II. The number of MVD in transplanted tumor of nude mice.

Groups	Control	5-FU	Repertaxin	Repertaxin + 5-FU
Mean	6.1	4.1	3.7	3.1
SD	1.9	1.4	1.6	1.7

Repertaxin treatment inhibits AKT and ERK1/2 phosphorylation in gastric cancer MKN45 cells. We next sought to determine which signaling pathways were activated downstream of CXCR1/2. Since AKT and ERK1/2 are well characterized regulators of cell growth, survival and invasion (46,47), we examined the effect of the CXCR1/2 inhibitor repertaxin on phosphorylation of these kinases. Treatment of gastric cancer MKN45 cells with either repertaxin alone or in combination with 5-FU for 48 h significantly downregulated phosphorylation of both AKT and ERK1/2 compared to control cells (Fig. 6E). These findings suggest that AKT and ERK1/2 signaling may be key downstream mediators of CXCR1/2 signaling in gastric cancer cells, and that inhibition of these kinases may be responsible, at least in part, for the

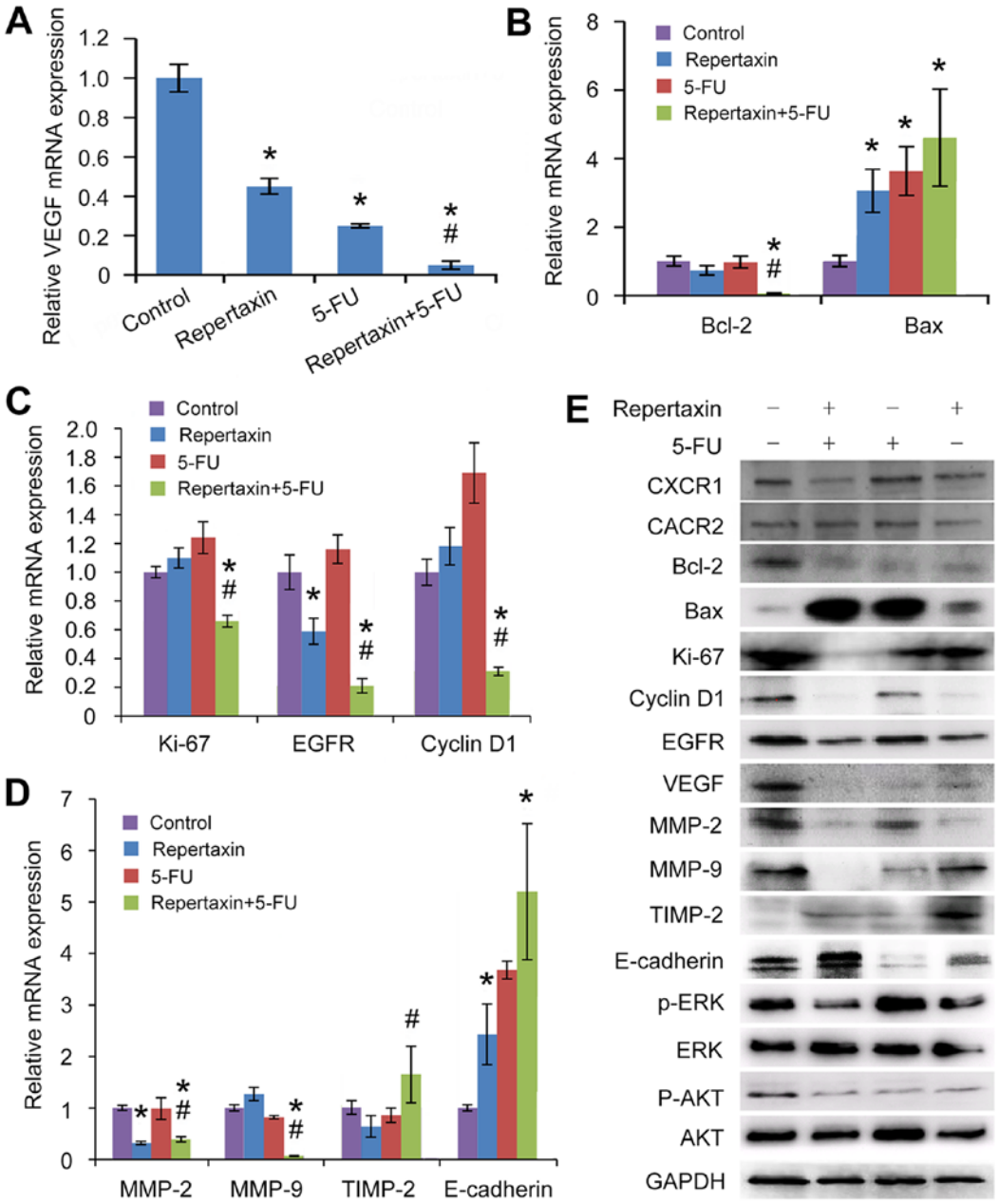

Figure 6. Effects of repertaxin and repertaxin combined with 5-FU on cell proliferation, cell cycle, cell apoptosis, cell migration and invasion-related signaling molecules in the MKN45 cells. MKN45 cells were treated with repertaxin (25 μg/ml) alone, 5-FU (10 μg/ml) alone, or combined repertaxin and 5-FU for 48 h. (A) angiogenesis (VEGF), (B) apoptosis (Bcl-2 and Bax), (C) proliferation and growth (cyclin D1, EGFR and Ki-67), (D) invasion and metastasis (MMP-9, MMP-2, TIMP-2 and E-cadherin). mRNA expression was determined by real-time RT-PCR. GAPDH was used as an internal control. (E) Cell lysates (50 μg) were fractionated by SDS-PAGE and subject to western blot analysis; GAPDH was used as a loading control. Data are shown as mean ± SD. *P<0.05 vs. control group; #P<0.05 combined treatment group vs. 5-FU alone group.

anti-proliferative, anti-invasive and anti-angiogenic activity of repertaxin.

Repertaxin combined with 5-FU alters expression of several proteins involved in cell cycle progression, apoptosis, migration and angiogenesis. We previously showed that repertaxin in combination with 5-FU induces apoptosis of MKN45 cells. To gain insight into this regulation, we examined the effect of these compounds on expression of key apoptosis regulators Bax and Bcl-2 (48). We found that repertaxin significantly decreased expression of the anti-apoptotic molecule Bcl-2 at both the mRNA and protein levels (Fig. 6B and E). In contrast, repertaxin increased expression of the pro-apoptotic molecule Bax (Fig. 6B and E). These findings suggest that repertaxin may influence apoptosis of gastric cancer cells by regulating the Bax/Bcl-2 ratio.

To better understand how repertaxin regulates proliferation of MKN45 cells, we examined expression of cyclin D1, EGFR and Ki-67. While repertaxin significantly decreased mRNA expression of EGFR (Fig. 6C), repertaxin alone had no effect on transcript levels of Ki-67 or cyclin D1 (Fig. 6C); in contrast, repertaxin was able to decrease expression of all three molecules at the protein level (Fig. 6E). Notably, repertaxin plus 5-FU significantly decreased expression of EGFR,

cyclin D1, and Ki-67 at both the mRNA and protein levels (Fig. 6C and E). These results suggest that repertaxin alone or in combination with 5-FU may induce cell cycle arrest and inhibit proliferation by reducing levels of cyclin D1, EGFR and Ki-67.

MMP-9, MMP-2, TIMP-2 and E-cadherin are all regulators of cellular migration and invasion. Repertaxin alone significantly decreased mRNA expression of MMP-2 and increased transcript levels of E-cadherin (Fig. 6D). Combination treatment with repertaxin and 5-FU significantly altered mRNA levels of MMP-2, MMP-9, TIMP-2 and E-cadherin (Fig. 6D). Consistent with these findings, protein levels of the molecules were similarly regulated by combined administration of repertaxin and 5-FU (Fig. 6E). Taken together, our data suggest that repertaxin and 5-FU may regulate gastric cancer cell migration and invasion via regulation of MMP-2, MMP-9, TIMP-2 and E-cadherin.

Finally, we examined the effect of repertaxin and 5-FU on expression of the key angiogenesis regulator VEGF. Expression of VEGF at both the mRNA (Fig. 6A) and protein (Fig. 6E) levels was most significantly decreased by combined treatment of repertaxin and 5-FU, suggesting that these compounds regulate angiogenesis by altering levels of VEGF in these cells.

Discussion

CXCR1/2 are expressed at high levels in a number of solid tumors, including gastric cancer. Chemokine signaling has been shown to regulate a number of tumorigenic processes, including proliferation, angiogenesis, survival, invasion and metastasis (13,20,49). However, the exact role of CXCR1/2 signaling in gastric cancer remains poorly characterized. In the present study, we show that gastric cancer MKN45 cells secrete IL-8, the ligand for CXCR1/2. Additionally, we show that small molecule inhibition of CXCR1/2 with repertaxin block many tumorigenic phenotypes of gastric cancer cells. Moreover, repertaxin can synergize with 5-FU, a commonly administered chemotherapy for gastric cancer treatment. Our data presented suggest that combined administration of repertaxin and 5-FU may be a useful therapeutic regimen for certain gastric cancer patients.

We found that repertaxin treatment decreases proliferation and colony-forming ability of MKN45 cells. This is consistent with other reports describing a role for chemokine signaling in various tumor types. We also find potentiated inhibition when repertaxin is combined with 5-FU. Although the exact mechanism of the enhanced effect of these compounds is unknown, it likely involves a combination of disrupted RNA synthesis (5-FU's primary mechanism of action) and CXCR1/2 blockade. However, much more work is required to completely understand this interaction. Interference with transcription is toxic to the cell, and we provide evidence here that inhibition of CXCR1/2 with repertaxin decreases expression of several molecules that regulate growth and survival. Thus, the combination of these two inhibitors is likely very toxic to cancer cells.

To begin to better understand the mechanism of action of these compounds, we examined expression of key downstream mediators of CXCR1/2 signaling. Previous study has shown that cells overexpressing CXCR1/2 exhibit increased levels of AKT phosphorylation (50). These findings suggest that AKT may act downstream of CXCR1/2 in gastric cancer cells as well. Additionally, IL-8-mediated activation of CXCR1/2 has been shown to activate phosphatidyl-inositol-3-kinase, which is upstream of AKT (51). CXCR1/2 signaling has also been shown to regulate the activity of MAPK signaling (51-53). Our previous data also verified that strong CXCR1/2 expression was positively associated with the phosphorylation of AKT and ERK1/2 (37). Thus, we examined the effect of repertaxin and 5-FU on phosphorylation of these molecules. We find that combined treatment of repertaxin and 5-FU significantly inhibited phosphorylation and activation of both AKT and ERK. Increased AKT expression is correlated with poor prognosis in tumors (47). Taken together, our data suggest that repertaxin and 5-FU may slow gastric cancer cell growth via inhibition of AKT and ERK signaling. This is in line with previous study, and also raises the possibility that AKT or ERK inhibitors may potentially be useful also in combination with repertaxin or 5-FU.

Furthermore, we found that repertaxin and 5-FU altered expression of several other molecules, such as Bax, Bcl-2, MMP-2, MMP-9, VEGF and E-cadherin, all of which are important regulators of apoptosis, migration and angiogenesis. It is currently unclear whether which, if any, of these downstream pathways are the essential mediators downstream of CXCR1/2 signaling in gastric cancer. More work is required to identify which pathways are most critical at mediating the inhibitory effects of repertaxin in these cells. The fact that we observed similar effects in mouse xenograft models treated *in vivo* suggests that this may represent a useful therapeutic strategy.

Although repertaxin and 5-FU had a more pronounced effect when administered together, the exact mechanism underlying this interaction is unknown. One possibility is that 5-FU when administered alone resulted in apoptosis and concomitant increase in IL-8 secretion. This secreted IL-8 could act through CXCR1/2 to protect tumor cells against chemotherapy (23). However, this would sensitize the cells to CXCR1/2 inhibition. Thus, in this setting, repertaxin would be more effective at inhibition of gastric cancer cell tumorigenesis. Although this represents one possible mechanism of action, further experimentation is necessary to determine the precise mechanism of action.

In summary, repertaxin alone or repertaxin in combination with 5-FU inhibits gastric cancer cell proliferation, survival, and migration both *in vitro* and *in vivo*. The present study suggests that targeting CXCR1/2 may represent a novel strategy for gastric cancer therapy, and that combined CXCR1/2 inhibition and 5-FU chemotherapy may be a useful therapeutic approach for certain gastric cancer patients.

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