Abstract. 1-Methylpropyl 2-imidazolyl disulfide (PX-12) has been proposed as an inhibitor of thioredoxin-1 (Trx-1) with anti-tumor activity. However, the antitumor activity of the Trx-1 redox signaling inhibitor PX-12 on colorectal cancer is still obscure. In the present study, we showed that PX-12 inhibited the growth of colorectal cancer DLD-1 and SW620 cells in a dose- and time-dependent manner. Further analysis demonstrated that PX-12 reduced cell colony formation and induced a G2/M phase arrest of the cell cycle. In addition, PX-12 treatment induced apoptosis, as observed by the increased number of Annexin V-positive cells and increased activation of caspase-3. Notably, a low dose of PX-12 inhibited colorectal cancer cell migration and invasion. Treatment of cancer cells with PX-12 reduced NOX1, CDH17 and S100A4 mRNA expression, and increased KLF17 mRNA expression. Moreover, PX-12 decreased S100A4 protein expression in the colorectal cancer cells. Collectively, the present study demonstrates the antitumor effects and therapeutic potential of PX-12 in colorectal cancer.

Introduction

Colorectal cancer is one of the leading causes of cancer-related mortality in most industrialized nations (1), and the effectiveness of conventional treatments for colorectal cancer is limited. Despite the fact that advances in the adjuvant treatment of colorectal cancer have been achieved for patients with lymph node-positive disease (2,3), the risk of recurrence is significantly high. Hence, extensive research has been conducted to find an effective adjuvant therapy for colorectal cancer (4), such as non-steroidal anti-inflammatory drugs, immunotherapy and gene therapy (5).

Thioredoxin (Trx) and thioredoxin reductase 1 (TR1) are among the major redox regulators in mammalian cells and control many cellular processes, including proliferation, defense against oxidative stress and apoptosis (6,7). Thioredoxin-1 (Trx-1), an ubiquitously expressed low-molecular-weight redox protein, is overexpressed in several types of human cancers (8-10) and has multiple effects in cells that include transactivating activity of a number of redox-sensitive transcription factors (11,12), and the regulation of DNA binding (13). Trx-1 has been shown to play a critical role in a series of human diseases including cancer of the cervix, pancreas, lung, colorectal and squamous cell cancer (8-10,14-16). Trx-1-transfected cells exhibit increased colony formation and cell growth in soft agar (17). Retrospective analyses in colorectal cancer indicate that increased expression of Trx-1 in cancer cells may be an independent prognostic factor (10).

1-Methylpropyl 2-imidazolyl disulfide (PX-12) is a potent inhibitor of Trx-1 by irreversible thioalkylation of Cys73 which lies outside the conserved redox catalytic site of Cys75 that inhibits the growth, migration and invasion of colorectal cancer cell lines.

Materials and methods

Cell lines and cell culture. Two colorectal cancer cell lines SW620 and DLD-1 were used in the present study. They were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 µg/ml streptomycin and 100 U/ml penicillin in a humidified 5% CO2 incubator at 37°C.

Cell viability and colony formation assays. Absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(MTT; Sigma-Aldrich) in living cells was measured to determine the effect of PX-12 on cell viability. Briefly, 1x10^4 cells/well were seeded in 96-well plates and exposed to the designated concentrations of PX-12 for the indicated times, MTT solution was added to each well. After the incubating time (4 h at 37˚C) was completed, the medium was withdrawn from the plates and 200 µl DMSO was added to each well to solubilize the formazan crystals. The optical density was measured at 490 nm using a Universal Microplate Reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA).

Cell cycle analysis. Cell cycle analysis was performed using propidium iodide (PI) (Ex/Em=488/617 nm; Sigma-Aldrich) staining. Briefly, 2x10^5 cells/well in 6-well plates were treated with 5 µM PX-12. At 24 h after the incubation time was completed, total cells, including floating cells, were then stained with Coomassie blue. The rate of colony formation was calculated with the following equation: Colony formation rate = (number of colonies/number of seeded cells) x 100%.

Apoptosis assay. For the apoptosis assay, apoptotic cell death was determined by Annexin V-FITC Apoptosis Detection Kit II (BD Pharmingen™, San Diego, CA, USA) according to the manufacturer's protocols. Briefly, 2x10^5 cells/well were seeded in 6-well plates and treated with 5 µM PX-12 for 48 h. After the incubation time was completed, total cells, including floating cells, were then washed with phosphate-buffered saline (PBS) and fixed in 70% (v/v) ethanol. The washing step was repeated again with PBS, and then staining with 50 µg/ml PI which contained 0.1% Triton X-100 and RNase (100 µg/l) was carried out. Cellular DNA content was measured using a FACSCalibur flow cytometer and analyzed using ModFit LT 2.0 software (both from Becton-Dickinson, Franklin Lakes, NJ, USA).

Migration and invasion assay. The effect of PX-12 on the migration and invasive abilities of the colorectal cancer cells in vitro was examined using Transwell assays. PX-12 (1 µM) which has little effect on cell growth at 24 h was used to treat the colorectal cancer cells. Migration and invasion assays were performed in Costar Transwell cell culture chamber inserts (Corning Costar Corp., Cambridge, MA, USA) with an 8-µm pore size. For the migration assay, a total of 1x10^5 cells were collected and seeded to the upper part of the chamber in 100 µl of serum-free medium. Medium with 10% FBS was added to the bottom chamber as a chemotactrant. Cells were supplemented with PX-12 during the incubation time (24 h at a concentration of 1 mM). After 24 h, the cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde, and stained with 0.1% of crystal violet. The number of cells that migrated to the lower surface of the membrane was counted in five random selected fields under a microscope at a magnification of x100. For the invasion assays, the polycarbonate membrane filters were coated with 1:3 diluted Matrigel (BD Biosciences, USA). All experiments were carried out in triplicate.

Reverse transcription (RT)-PCR. Total RNA was extracted from cells using TRIzol (Invitrogen), and reverse-transcribed into cDNA using a SuperScript II First Strand DNA Synthesis Kit II (BD Pharmingen™, San Diego, CA, USA). The protein concentration was determined using the BCA protein assay kit (Qcbio Science & Technologies Co., Ltd., Shanghai, China). Total proteins (30 µg) were electrophoresed on a 12% SDS-PAGE and transferred onto PVDF membranes. The membranes were then incubated with an appropriate dilution of the primary antibody for 1 h followed by incubation with the horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL, USA), and visualized using the enhanced chemiluminescence substrate kit (Amersham Biosciences, Inc.). The primary antibodies used in the present study were polyclonal antibodies against S100A4 and actin (80-50; Abcam, Cambridge, UK).

Statistical analysis. The results in the present study are expressed as means ± SD. Statistical analysis was performed...
Results

PX-12 inhibits colorectal cancer cell proliferation and colony formation. We first examined the effect of PX-12 on the cell viability of human colorectal cancer cell lines DLD-1 and SW620 using an MTT assay. After exposure to a range of concentrations of PX-12 (0, 1, 2.5, 5, 7.5, 10 µM) for 48 h, the viability of the DLD-1 and SW620 cells was reduced in a dose-dependent manner (Fig. 1A). In addition, a time-dependent decrease in cell viability was also observed when cells were treated with 5 µM PX-12 (Fig. 1B). As shown in Fig. 1C, DLD-1 and SW620 cells treated with the indicated concentrations of PX-12 for 14 days showed a significant reduction in the colony formation in a dose-dependent manner.

PX-12 alters the cell cycle distribution and induces cell apoptosis in colorectal cancer cells. To evaluate the effects of PX-12 on the cell cycle, cell cycle distributions were determined using flow cytometry. After the indicated PX-12 treatments for 24 h, PX-12 significantly induced G2/M phase arrest of the DLD-1 and SW620 cells (Fig. 2). Cell apoptosis was detected by Annexin V-FITC/PI staining. We found that PX-12 induced DLD-1 and SW620 cell apoptosis in a dose-dependent manner (Fig. 3A and B). In addition, PX-12 increased the levels of activated caspase-3 expression in the SW620 and DLD-1 cells at the concentration of 10 µM PX-12 for 48 h (Fig. 3C and D).

PX-12 inhibits colorectal cancer cell migration and invasion. The effects of PX-12 on the migration and invasion of the colorectal cancer cells were evaluated using Transwell filter membrane chambers. PX-12 (1 µM), a dose with no significant effect on the rate of apoptosis, was used in the migration and invasion study. As shown by the representative images in Fig. 4A and B, the migration and invasion abilities were inhibited in the PX-12-treated colorectal cancer DLD-1 and SW620 cells. PX-12 treatment of the DLD-1 and SW620 cells decreased the cell counts in the migration assay by 78 and 83% respectively, and in the invasion assay by 72 and 98.6%, respectively.
Figure 2. PX-12 alters the cell cycle distribution in SW620 and DLD-1 colorectal cancer cells. (A and C) Representative results are shown for the SW620 and DLD-1 cells treated with 5 µM PX-12 for 48 h. (B and D) After treatment with the indicated concentrations of PX-12 for 48 h, cell cycle distribution was analyzed using flow cytometry. Each value represents the mean ± SD of three independent experiments. PX-12, 1-methylpropyl 2-imidazolyl disulfide.

Figure 3. PX-12 induces colorectal cancer cell apoptosis. DLD-1 and SW620 cells were treated with various concentrations of PX-12 for 48 h, and cell apoptosis was detected by Annexin V-FITC/PI staining. (A) Representative results are shown for the DLD-1 and SW620 cells treated with various concentrations of PX-12 for 48 h. (B) The percentage of Annexin V-positive cells was calculated 48 h after treatment with increasing concentrations of PX-12 (1-10 µM). Data are presented as the mean ± SD of three independent experiments. (C) SW620 cells were treated with 10 µM PX-12 for 48 h, and the level of caspase-3 expression was detected using flow cytometry. (D) DLD-1 cells were treated with 10 µM PX-12 for 48 h, and the level of caspase-3 expression was detected using flow cytometry. PX-12, 1-methylpropyl 2-imidazolyl disulfide.
Effects of PX-12 on the NOX1, CDH17, KLF17, S100P and S100A4 mRNA expression in colorectal cancer cells. To examine the effect of PX-12 on NOX1, CDH17, KLF17, S100P and S100A4 mRNA levels in colorectal cancer DLD-1 and
SW620 cells, real-time PCR analysis was performed. As shown in Fig. 5A, levels of NOX1, CDH17, S100P and S100A4 mRNA expression were reduced by >50%, and KLF17 mRNA expression was increased after treatment with 2.5 µM PX-12 for 48 h in the DLD-1 cells. In another colorectal cancer cell line SW620, most of the results were similar as those in the DLD-1 cells except for S100P mRNA expression which had no significance change after treatment with PX-12.

**PX-12 decreases S100A4 protein expression in colorectal cancer cells.** To examine the effect of PX-12 on S100A4 protein in colorectal cancer cells, western blot analysis was performed. As shown in Fig. 5B, the S100A4 protein expression was diminished as the concentration of PX-12 increased in the SW620 cells.

**Discussion**

The differentiation and proliferation of colorectal cancer cells are complex events and the relevant mechanisms have been universally investigated (21-23). Trx-1 expression was reported to be increased in several types of human cancers, including colorectal carcinoma (10,24). Trx-1 has various biological activities, such as antioxidant, growth control, anti-apoptotic properties and inflammation regulation that provide reducing equivalents and a transcriptional regulator (6,25,26). PX-12, an inhibitor of Trx-1, has shown excellent in vitro antitumor activity, and Ramanathan et al reported the first-in-human phase I trial of PX-12 in patients with advanced solid tumors (20). However, the antitumor effect of PX-12 in colorectal cancer cells, particularly the effect of PX-12 on cancer cell migration and invasion, is still obscure.

The aim of the present study was to assess the effects of PX-12 on colorectal cancer cells in vitro. Following exposure to a range of concentrations of PX-12 (0, 1, 2.5, 5, 7.5 and 10 µM) for 48 h, cell growth was inhibited as the concentration of PX-12 increased. The same result was shown when the exposure time was prolonged. We also demonstrated that PX-12 inhibited colorectal cancer cell colony formation. The susceptibility of colorectal cancer cells to PX-12 appeared to significantly increase after the incubation time was prolonged or the concentration of PX-12 was increased. Namely the inhibitory effects of PX-12 on colorectal cancer cells were dose- and time-dependent. DNA flow cytometric analysis indicated that PX-12 significantly induced G2/M phase arrest of the cell cycle in colorectal cancer DLD-1 and SW620 cells. Similarly, PX-12 induced G2/M phase arrest in breast cancer and lung cancer cells (27,28). Therefore, this body of evidence suggests that the G2/M phase arrest in PX-12-treated cells is an underlying mechanism to suppress the growth of cancer cells, including colorectal cancer cells.

In the apoptosis assay, PX-12 also increased the number of Annexin V-positive cells at the designated concentrations of PX-12 for 48 h, suggesting that PX-12-induced colorectal cancer cell death occurred via apoptosis. Our results indicated that PX-12 induced apoptosis in human colorectal cancer cells through enhancement of caspase-3 activation. In addition, the migration and invasion abilities were inhibited in the PX-12-treated colorectal cancer cells. To further elucidate the molecular mechanisms of the antitumor effects of PX-12, we performed gene array studies and comparative gene profiling analysis of DLD-1 cells treated with PX-12 (data not shown). NOX1, CDH17, KLF17, S100P and S100A4 were found to be regulated by PX-12 in the DLD-1 cells. NOX1, an NADPH oxidase, which is known to enhance reactive oxygen species (ROS), is expressed in all types of tissues including osteoclasts, vascular smooth muscle, and normal gastric and colonic mucosa (29); there is growing evidence that Nox1 is overexpressed in many human colon adenocarcinomas (30). CDH17 was found to increase cell adhesion and proliferation in colon cancer cells, by regulating α2β1 integrin signaling to induce particular focal adhesion kinase and Ras activation (31). KLF17 plays vital roles in many oncogenic processes, such as tumor growth and metastasis. Studies have reported that KLF17 acts as an inhibitor of epithelial-to-mesenchymal transition and metastasis in breast cancer (32). More and more evidence suggests that S100P plays a critical role in tumors. This gene was found to be expressed in several different types of cancer, including colon, pancreas, breast and lung cancer (33-36). Recent studies have also shown that S100A4, a small Cu2+ binding protein, is highly expressed in different tumor tissues (37). High S100A4 expression is related to increased metastasis formation in tumors (38), including colorectal cancer (39,40). We further confirmed a change in the expression of genes by quantitative RT-PCR. After exposure of DLD-1 and SW620 cells to PX-12 for 48 h, NOX1, CDH17 and S100A4 mRNA expression was reduced, while KLF17 mRNA expression was increased. This indicated that PX-12 may trigger the inhibition of NOX1, CDH17 and S100A4 mRNA expression and an increase in KLF17 mRNA expression. S100A4 is overexpressed in a range of different tumor types, and plays an important role in the process of cancer metastasis (38,41). S100A4 expression is significantly associated with positive lymph node metastasis in colorectal cancer (39), and targeting the expression of S100A4 with calcimycin was found to inhibit colon cancer cell migration and invasion (42,43). As shown in Fig. 5B, the S100A4 protein expression was diminished in the PX-12-treated SW620 cells. The mechanism involved in the decrease in S100A4 protein by PX-12 is not known. It is known that PX-12 is an inhibitor of Trx-1 and has antitumor activity. One may predict that inhibition of the Trx-1 redox system leads to decreased S100A4 protein and trans-activation of downstream targets. Therefore, future research is required to investigate the correlation of the antitumor effect of PX-12 on colorectal cancer cells with NOX1, CDH17, S100A4, S100P and KLF17 expression.

In summary, we demonstrated that PX-12 inhibited the growth of colorectal cancer cells via G2/M phase arrest and induction of apoptosis. Notably, a low dose of PX-12 inhibited colorectal cancer cell migration and invasion. PX-12 has been shown to have significant antitumor activity in colorectal cancer in vitro. This antitumor effect may be associated with NOX1, CDH17, S100A4 and KLF17 mRNA expression. The present study provides important insight into the antitumor effects of PX-12 on colorectal cancer cells and represents a novel and effective strategy for treating cancer.

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