PC-407 inhibited proliferation and induced apoptosis in human colon cancer SW-1116 cells

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ABSTRACT

AIM: To study whether PC-407 [4-[5-naphthyl-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide] inhibits cell viability and induces apoptosis in human colon cancer SW-1116 cells. METHODS: Inhibition of SW-1116 proliferation was measured by MTT assay. Morphological assessment of apoptosis was performed with fluorescence microscope and electron microscope. DNA fragmentation was visualized by agarose gel electrophoresis. The amount of apoptotic cells was measured by flow cytometry. RESULTS: PC-407 inhibited SW-1116 cell proliferation in a concentration-dependent manner after 3 d of treatment, and the IC50 for PC-407 inhibition of cell number was 16.67±0.17 µmol/L. After incubation of SW-1116 cells with PC-407 20 µmol/L for 24 h, morphological changes of typical apoptosis were observed by AO/EB staining or transmission electron microscopy. Flow cytometry analysis showed that PC-407 induced apoptosis in SW-1116 cells in a time- and concentration-dependent manner. The agarose gel electrophoresis of DNA revealed a “ladder” pattern 48 h later. CONCLUSION: PC-407 inhibited proliferation and induced apoptosis in the human colon cancer SW-1116 cell line.

INTRODUCTION

The dietary habits of China’s population have changed greatly in recent years, particularly in the area of foods high in fat content, giving rise to an increase in the incidence of colon cancer. A dramatic development in oncology in the past decade was the recognition that nonsteroidal anti-inflammatory drugs (NSAIDs) share the property of inhibiting the COX enzymes and can delay or prevent certain kinds of cancer, including colon cancer[1]. Therefore, much hope is currently placed on chemo-prevention of colon cancer. Cyclooxygenases (COX) are key enzymes in the conversion of arachidonic acid (AA) to prostaglandins (PGs). In the classical hypothesis, there are two isoforms of COX, COX-1 and -2, which are very similar in structure, but quite different in their expression pattern and function within organisms[2,3]. COX-1 is constitutively expressed in many cells and tissues, and is responsible for maintaining gastric mucosal integrity. COX-2 is induced by a variety of stimuli such as cytokines, growth factors, and tumor promoters[4], and plays an important role in mediating inflammation. In addition, COX-2 expression levels are increased in colon cancer tissues.

Numerous epidemiological studies indicate that chronic use of NSAIDs lowers the mortality rate from colorectal cancer. However, prolonged use of classical NSAIDs results in untoward gastrointestinal side effects because they inhibit COX-2 and COX-1 simultaneously. The latter can lead to bleeding or ulceration in the gastrointestinal tract. Thus, COX-2 selective inhibitors are becoming a very promising target in this field. Celecoxib, a COX-2 selective inhibitor, shows a chemo-preventive effect both in animal tumor

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models and in patients, and is the only NSAID used for the prevention of colorectal cancer in patients with familial adenomatous polyposis (FAP) so far\cite{5}.

PC-407 is a compound designated as 4-[5-naphthyl-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide based on the chemical structure of celecoxib. Its empirical formula is C_{20}H_{14}F_{3}N_{3}O_{2}S, and the molecular weight is 417. Our previous study showed the antitumor effects of celecoxib on colon carcinoma cells\cite{6}, therefore the present study is designed to study whether PC-407, a new candidate compound of COX-2 selective inhibitor can inhibit cell viability and induce apoptosis in human colon cancer SW-1116 cells.

**MATERIALS AND METHODS**

**Reagents** Celecoxib and PC-407 were synthesized in our lab, with a purity of 99.5 % and 97.5 % respectively. PC-407 was a new candidate compound of COX-2 selective inhibitors previously reported by us\cite{7,8}, which was white granular crystal and liposoluble with a melting point of 188.7-189.0 ºC. Trypan Blue, MTT, AO (acridine orange), and EB (ethidium bromide) were acquired from Sigma. Me_{2}SO (Sigma) was used as a solvent. Concentrated drug stocks were diluted in RPMI-1640 (Gibco) medium before addition to cell cultures. The final Me_{2}SO concentration in cultures was kept at 0.1 %.

**Cell culture** The human colon cancer cell line SW-1116 was obtained from Xi-an Cell Engineering Center (Xi-an, China). SW-1116 cells were grown in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS was from Tianjin China), benzylpenicillin 100 kU/L, and streptomycin 100 mg/L. The cells were incubated at 37 ºC in a humidified atmosphere at 5 % CO_{2}.

**Assessment of cell proliferation** Cells were plated at a density of 5×10^{4} cells/well in a 800 µL volume into 24-well plates from Costar (New York, USA). The IC_{50} of celecoxib for inhibition of cell viability was about 40 µmol/L in other colon cells\cite{7}. To observe the trend of cell growth clearly, a concentration which was a little lower than 40 µmol/L was used in our experiments. Cells were treated with celecoxib 30 µmol/L or PC-407 30 µmol/L. Control cells had an amount of Me_{2}SO added equivalent to the drug-treated cells (0.1 %, v/v), and each group had three wells repeated. Cells were counted every 24 h for one week. All the cells were suspended and exposed to 0.4 % (w/v) Trypan Blue at 37 ºC for 1 min to distinguish viable cells from non-viable cells. Living cells did not take up the dye, while dead cells did. Living cells were counted by a hemacytometer.

Cells were seeded at a density of 5×10^{5} cells/well in a 100 µL volume of medium in 96-well plates and allowed to attach overnight. The cells were then treated with various concentrations of drugs while the experimental controls were treated with 0.1 % (v/v) Me_{2}SO only. MTT (5 g/L) 10 µL was added 72 h later. After incubation at 37 ºC for 4 h, the supernatant was aspirated, and 150 µL Me_{2}SO was added to each well. The negative control well contained medium only. Absorbance was measured at 570 nm by a 96-well spectrophotometric plate reader.

**Morphological study** Exponential growth phase SW-1116 cells were treated with PC-407 20 µmol/L for 24 h or 48 h, and all the floating cells and the attached cells were harvested with 0.25 % (w/v) trypsinase. The cell suspension 95 µL was mixed with 5 µL of dye mixture containing AO (100 mg/L) and EB (100 mg/L) in PBS. The cells were observed immediately by a fluorescence microscope. The peak excitation wave length was 490 nm.

Cells were treated with PC-407 20 µmol/L for 48 h, then all the cells in the culture flask were harvested. After being washed with 0.1 mol/L PBS twice, the cells were centrifuged and fixed in glutaraldehyde for observation through a transmission electron-microscopy.

**DNA content flow cytometric analysis** For DNA content analysis, cells were treated with PC-407 20 µmol/L and harvested at 6 h, 24 h, and 48 h. Cells (1×10^{5}) were washed in ice cold PBS and centrifuged (1000×g), then fixed with 70 % ice cold ethanol and incubated at 4 ºC for at least 30 min. After the cells were washed twice with cold PBS, they were re-suspended in PI master mix at a final cell density and incubated at 37 ºC for 30 min in darkness. DNA content of
cells was measured by a flow cytometer (ELTTE ESP, BACKMAN USA).

**Dual parameter flow cytometry analysis** Cells were stained with fluorescein isothiocyanate (FITC) labeled annexin-V, and simultaneously with PI stain, to discriminate intact cells (annexin−/PI−) from apoptotic cells (annexin+/PI−) and necrotic cells (annexin+/PI+). After the cells were treated with PC-407 20 µmol/L, 40 µmol/L, and 100 µmol/L for 24 h, they (1×10⁶) were washed twice with ice cold PBS and incubated for 30 min in a binding buffer of 1 mg/L PI and 1 mg/L FITC-labeled annexin-V. FACS analysis for annexin-V and PI staining was performed by the same flow cytometer mentioned above.

**DNA fragmentation assay** Cells (1×10⁶) were treated with 0.1 % (v/v) Me₂SO (control), PC-407 20 µmol/L and 40 µmol/L for 48 h. According to the manufacturer’s protocol, DNA fragments were extracted using a DNA Mini-Prep Kit (V-Gene Biotechnology Limited). Then 10 µL DNA samples were mixed with 4 µL loading dye (Sigma) and analyzed by a 1.8 % agarose gel (Roche Diagnostics) pre-stained with 1 mmol/L EB (Sigma). Each gel electrophoresis included a DNA marker (100-2000 bp).

**Statistical analysis** Data obtained represented mean values of at least three different experiments and expressed as the mean±SD and analyzed by t-test. P<0.05 was considered to be statistically significant.

**RESULTS**

**Growth-inhibitory effects** Compared with celecoxib, the ability of PC-407 to inhibit the growth of SW-1116 cells was much more potent. After three days, the number of cells in the sample treated with PC-407 30 µmol/L decreased, while the number of cells treated with celecoxib 30 µmol/L gradually increased (Fig 1A). Subsequently, MTT assay demonstrated that after 72 h of treatment the IC₅₀ for PC-407 inhibition of cell viability was 16.67±0.17 µmol/L, and that of celecoxib was 33.6±1.8 µmol/L (Fig 1B).

**Morphological changes** When SW-1116 cells were treated with PC-407 20 µmol/L for 24 h, the morphological features of apoptotic cells, such as cell surface protuberances and nuclear fragment, were identified by AO staining by a florescence microscope. After 48 h, the typical apoptotic body appeared, and the late apoptotic cells were observed by EB staining (Fig 2). The apoptotic phenomenon was further demonstrated by transmission electron-microscopy (Fig 3).

**Flow cytometric analysis** After cells were treated with PC-407 20 µmol/L for 6 h, 24 h and 48 h, 6.6 %, 16.7 %, and 25.7 % of apoptotic cells were located in sub-G1-phase position respectively, which indicated that PC-407 induced apoptosis in SW-1116 cells in a time-dependent manner (Fig 4). The dual parameter flow cytometry analysis revealed that PC-407 caused apoptosis in the SW-1116 human cells in a concentration-dependent manner. After cells were exposed to PC-407 for 24 h (20, 40, or 100 µmol/L), 10.8 %, 20.5 %, and 38.4 % cells were undergoing apoptosis (Fig 5).

**DNA fragmentation** After treatment with PC-407 for 48 h, DNA electrophoresis of SW-1116 cells showed a typical DNA “ladder” of apoptosis (Fig 6).
DNA fragment gel electrophoresis was used as a marker of end-stage apoptosis to confirm the induction of apoptosis by PC407 in SW1116 cells.

A number of studies have shown the inhibition of COX inhibitors on the growth of tumor cells both in vitro and in vivo\cite{11-14}. COX-2 selective inhibitors have become more popular as they have fewer side effects on the dying cells. Such cleavage is initially digested into 300 and/or 50 kbp fragments followed by more complete digestion into 180-200 bp integer multiples, the hallmark of apoptosis\cite{9,10}. DNA fragment gel electrophoresis was used as a marker of end-stage apoptosis to confirm the induction of apoptosis by PC407 in SW1116 cells.

**DISCUSSION**

Apoptosis is known as programmed cell death. In cancer cells, the incidence of apoptosis and the rate of cell proliferation are uncontrolled, which causes tumor invasion. It is a standard practice to induce carcinoma cells to undergo apoptosis by various anticancer agents in cancer chemotherapy. Apoptosis is a typical phenomenon in cell death, and is morphologically distinct from necrosis. The chromatin condensation during apoptosis may be a consequence of DNA cleavage within the dying cells. Such cleavage is initially digested into 300 and/or 50 kbp fragments followed by more complete digestion into 180-200 bp integer multiples, the hallmark of apoptosis\cite{9,10}. DNA fragment gel electrophoresis was used as a marker of end-stage apoptosis to confirm the induction of apoptosis by PC407 in SW1116 cells.

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For example, PGE$_2$, a kind of PGs, exerted especially carcinogenic effects in the human body$^{[15,16]}$. On the other hand, more conflicting evidence had appeared as some groups indicated that NSAIDs, including COX-2 inhibitors, kept a COX-independent effects manner in cancers$^{[17,18]}$. Their evidence showed that the dose of NSAIDs used was much higher than that needed to inhibit COX-2 enzymatic activity, and NSAIDs were effective in a COX-2 negative cell line$^{[19]}$. Our experiments demonstrated that celecoxib and PC-407 inhibited proliferation and induced apoptosis in human colon cancer cell line SW-1116 effectively when using a high dose ($\geq 10$ µmol/L). This suggested that there was a close relationship between COX and colon cancer because of NSAIDs mechanisms of action through the inhibition of COX. In recent years, more and more researchers have been actively charting the apoptotic pathways of NSAIDs in cancer. There are still tough issues that need to be resolved before applying NSAIDs into the treatment of colon cancer in a clinical setting. It needs much more investigations to make clear the crux of NSAIDs and their role in the treatment of colon cancer.

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