Cancer Letters 335 (2013) 479–486

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Synergistic antitumor effect of 5-fluorouracil in combination with parthenolide in human colorectal cancer

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ARTICLE INFO

Article history:
Received 17 December 2012
Received in revised form 8 March 2013
Accepted 8 March 2013

Keywords:
5-Fluorouracil
Parthenolide
Combination therapy
Anti-cancer
Colorectal cancer

ABSTRACT

Parthenolide (PT), a NF-κB inhibitor, has recently been demonstrated as a promising anticancer agent that promotes apoptosis of cancer cells. 5-fluorouracil (5-FU) has been a drug of choice for treatment of colorectal cancer (CRC). Unfortunately, many of the therapies that use 5-FU alone or in combination with other agents are likely to become ineffective due to drug resistance. In the present study, we investigated the antitumor effect of PT combined with 5-FU on a human CRC cell line, SW620. The results demonstrated that combination of PT and 5-FU induced apoptosis which was determined using MTT, cell cycle analysis, annexin-V assay, and Hoechst 33258 staining. Apoptosis through the mitochondrial pathway was confirmed by detecting regulation of Bcl-2 family members, cytochrome C release, and activation of caspase 3 and 9. Moreover, intra-peritoneal injection of PT and 5-FU showed significant inhibition of tumor growth in the xenograft model. These results demonstrate that PT exhibits anticancer activity in human colorectal cancer *in vitro* and *in vivo*. These findings provide an efficacious strategy to overcome 5-FU resistance in certain CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide [1]. Other than surgery, treatment for CRC patients relies primarily on chemotherapy, especially the patients with advanced CRC. Among the chemotherapeutic agents for CRC, 5-fluorouracil (5-FU), which is a classical chemotherapy agent, has been the first line regimen for treating CRC over several decades [2,3]. 5-FU is known to block DNA synthesis by the inhibition of thymidylate synthase (TS) that is regulated by cell cycle proteins controlled by phosphorylation [4]. The antitumor effect of 5-FU is due to the induction of apoptosis resulting from the regulation of several molecules, such as Bax, relative to Bcl-2 or Bcl-xL in CRC cells [5]. However, the clinical insufficiency appears to be caused from resistance of 5-FU and severe side effects. Therefore, 5-FU is usually used for combined therapy with other antineoplastic agents and radiation to enhance its antitumor effect. Representative combined therapy with 5-FU, named FOLFOX regimen (5-FU, leucovorin, and oxaliplatin), has widely been used for CRC patients. Although efficacy of 5-FU is enhanced by FOLFOX regimen, resistance of 5-FU is still recognized as a reason for CRC therapy failure. Therefore, a novel chemotherapy combination still needs to be explored.

Parthenolide (PT), the natural product, has been used for the treatment of fever and inflammatory disease [6]. Over two decades, it is known that PT induces apoptosis in various cancer cells, including human hepatocellular carcinoma cells, human lung cancer cells, human stomach cancer cells, and glioblastoma cells [7–11]. Apoptotic effect of PT has confirmed that it is associated with inhibition of the nuclear factor (NF)-κB [9,12], the signal transducer and activator of transcription 3 (STAT3) [7], with enhanced oxidative stress [13], and mitochondria-mediated pathway [14]. In our previous study, we have found that PT effectively induce apoptosis in CRC cells through mitochondrial dysfunction [15]. We have also demonstrated that PT could be a potential chemopreventive and therapeutic agent for CRC using xenograft models [15]. Various investigations of combined therapy using PT are reported in recent years: PT sensitizes cancer cells to the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), nonsteroidal anti-inflammatory drugs (NSAIDs), anticancer drugs toxicity, and radiation [7,13,16–20]. However, there are no studies that have examined the improvement of 5-FU resistance by combining with PT in CRC.

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http://dx.doi.org/10.1016/j.canlet.2013.03.007

In the present study, we investigated whether 5-FU and PT have synergistic action in CRC cells, more importantly, whether this results in a synergistic inhibition of CRC cell growth *in vivo*. Thus, our objective was to evaluate the potential of combination therapy with 5-FUand PT for the treatment of CRC.

2. Materials and methods

2.1. Chemicals and reagents

Parthenolide and z-VAD-FMK were from Calbiochem (San Diego, CA). 5-FU was purchased from Sigma (St. Louis, MO, USA). Annexin-V FITC was from Invitrogen (Eugene, OR). TUNEL assay kit was from Promega (Madison, WI). Anti-Bcl2, anti-Bid, anti-Bid, anti-Bax, anti-cytochrome c, anti-caspase 3, anti-caspase 9, anti-p53, and anti-actin antibody were from Santa Cruz Technology (Beverly, MA).

2.2. Cell culture and treatment

A human colorectal cancer cell line, SW620 cells (American Type Culture Collection, Rockville, MD) was cultured in the RPMI 1640 medium supplemented with 10% FBS, 100 units penicillin, and 100 units streptomycin. For the treatment of cells with PT or 5-FU, cells were sub-cultured in RPMI 1640 medium without FBS for 12 h. PT and 5-FU were dissolved in DMSO as a stock solution at 100 mM and diluted with FBS-free medium to achieve designated concentrations. Same concentration of DMSO was applied to cells as a control.

2.3. MTT colorimetric survival assay

SW620 cells were plated at a density of 1.0×10^4 cells per well in 96 well plates. Cells were treated with single or dual agents for 24 h, and then the medium was removed and 200 μl of fresh medium plus 20 μl of 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT, 2.5 mg dissolved in 50 μl of dimethylsulfoxide, Sigma, St. Louis, MO) were added to each well. After incubation for 4 h at 37 °C, the culture medium containing MTT was withdrawn and 200 μl of dimethylsulfoxide (DMSO) was added, followed by shaking until the crystals were dissolved. Viable cells were detected by measuring absorbance at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

2.4. Cell cycle and sub-G1 analysis

Cell cycle and sub-G1 distribution were determined by the staining of DNA with propidium iodide (PI; Sigma–Aldrich) (Ex/Em = 488 nm/617 nm). PI is a fluorescent biomolecule that can be used to stain DNA. In brief, 1×10^6 cells were incubated with single or dual agents for 24 h. Cells were then washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight. Cells were washed again with PBS and then incubated with PI (10 μg/ml) with simultaneous treatment of RNase at 37 °C for 1 h. The percentages of cells in different phases of the cell cycle or having sub-G1 DNA content were measured with a BD LSR flow cytometer and analyzed using Cell Quest software (Becton Dickinson, NY).

2.5. Apoptosis assay by annexin V-FITC and Hoechst 33258

After being incubated with single or dual agent for 24 h, the cells were trypsinized, collected, washed with ice-cold PBS, suspended in a 500 °μl annexin V binding buffer containing 5 μl of annexin V-FITC, and incubated for 15 min at room temperature in the dark. The fluorescence was measured using a BD LSR flow cytometer and processed with Cell Quest software for analysis. Apoptotic feature of cancer cells was assessed by DNA condensation using Hoechst 33258. The cells were treated with single of dual agents for 24 h, and then stained with Hoechst 33258 (1 μg/ml) at 37 °C for 10 min. Nuclear morphology was examined under a Confocal Laser Scanning Microscope (Carl Zeiss, Germany) to identify cells undergoing apoptosis.

2.6. Cell extraction and western blotting

Cells were collected, washed twice with PBS, and then lysed for 30 min on ice in a lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM EDTA, 1% TritonX-100, 0.5% SDS and protease inhibitor cocktail). The protein concentration in cell lysates was measured by using Protein Quantification kit from Bio-Rad. Total 50 μg proteins were loaded onto a SDS–PAGE gel. After transferring and blocking, the membrane was probed with various antibodies (anti-Bcl2, anti-Bcl-xL, anti-Bax, anti-Bid, anti-cytochrome c, anti-caspase 3, anti-caspase 9, anti-p53 and anti-actin). The signal was detected by using enhanced Westone (Intron, Daejeon, Korea), captured, and analyzed by a Luminescent Image Analyzer (LAS-3000, Fuji Film, Japan).

2.7. Xenograft models

Animal experiment was approved by the Chonbuk National University Animal Care and Use Committee (Approved Number: CBU 2012-0035). SW620 (6×10^6) cells were injected into nude mice. Mice were randomized and assigned to control group and treatment group and intraperitoneally injected three times a week vehicle (DMSO), 2.5 mg/kg PT, 30 mg/kg 5-FU and combination of 2.5 mg/kg PT and 30 mg/kg 5-FU, respectively. Single or dual treatment was started on 5 days after tumor cell implantation (0.5 mm^3 tumor volume). Tumor diameters were measured three times a week, and tumor volumes were also calculated (volume = length × width × height × π/6). The experiment was terminated on 28 days, and the tumors were harvested for TUNEL assay.

2.8. TUNEL assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay was carried out in paraffin-embedded tissue sections (5 μm). Apoptotic cells on tissue were measured quantitatively using an ApopTag in situ Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Four fields at 40 × magnification were selected at the proliferation front of each tumor, and TUNEL-positive cells were counted.

2.9. Statistical analysis

The data are presented as a mean ± SE of at least three independent experiments done in duplicate. Representative blots are shown. All the data was entered into the Microsoft Excel 5.0, and SPSS Software was used to perform the two-tailed *t* tests or the analysis of the variance, where appropriate. *P* values < 0.05 were considered significant.

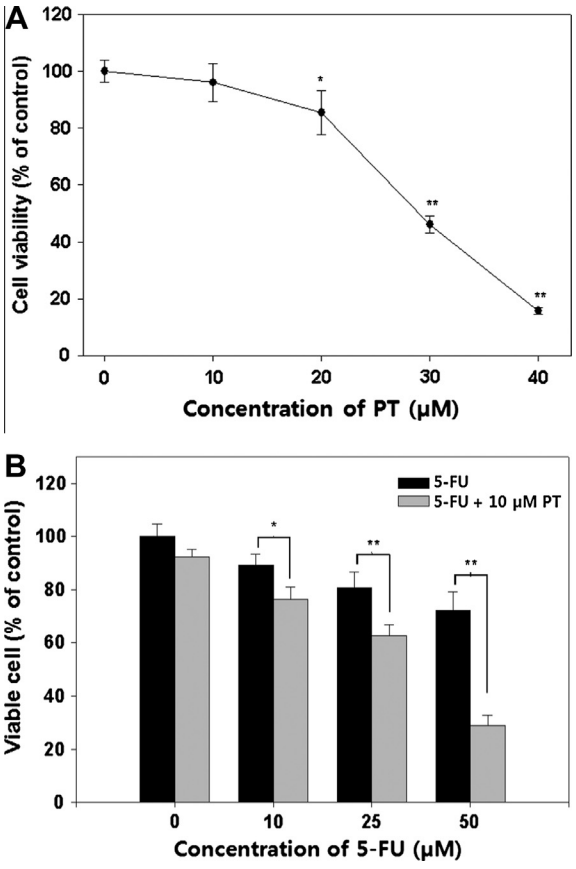

Fig. 1. Inhibitory effect induced by combination of PT and 5-FU on growth. (A) The cells were treated with PT with various concentrations (0, 10, 20, 30 and 40 μM) for 24 h. Data represent the mean values ± SD of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control. (B) The cells were treated with 5-FU alone or combination of 5-FU and 10 μM PT for 24 h. Data represent the mean values ± SD of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the cells treated 5-FU alone.