Synergism of Cytotoxicity Effects of Triptolide and Artesunate Combination Treatment in Pancreatic Cancer Cell Lines

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Abstract

Background: Triptolide, extracted from the herb Tripterygium wilfordii Hook.f that has long been used as a natural medicine in China, has attracted much interest for its anti-cancer effects against some kinds of tumours in recent years. Artesunate, extracted from the Chinese herb Artemisia annua, has proven to be effective and safe as an anti-malarial drug that possesses anticancer potential. The present study attempted to clarify if triptolide enhances artesunate-induced cytotoxicity in pancreatic cancer cell lines in vitro and in vivo. Methods: In vitro, to test synergic actions, cell viability and apoptosis were analyzed after treatment of pancreatic cancer cell lines with the two agents singly or in combination. The molecular mechanisms of apoptotic effects were also explored using qRT-PCR and Western blotting. In vivo, a tumor xenograft model was established in nude mice, for assessment of inhibitory effects of triptolide and artesunate. Results: We could show that the combination of triptolide and artesunate could inhibit pancreatic cancer cell line growth, and induce apoptosis, accompanied by expression of HSP 20 and HSP 27, indicating important roles in the synergic effects. Moreover, tumor growth was decreased with triptolide and artesunate synergy. Conclusion: Our result indicated that triptolide and artesunate in combination at low concentrations can exert synergistic anti-tumor effects in pancreatic cancer cells with potential clinical applications.

Keywords: Triptolide - artesunate - pancreatic cancer cell lines - cytotoxicity - synergism

Introduction

Pancreatic cancer is a malignant neoplasm originating from transformed cells arising in pancreas, one of the highest fatality rates of all cancers, extremely poor prognosis and resistant to current chemotherapies, therefore, new strategies or reagents to tackle this disease are needed.

Triptolide (TPL) is a diterpenoid triepoxide and the principal active ingredient of Tripterygium wilfordii Hook. f. that has been used as a natural medicine in China for hundreds of years, which has pharmacological and biochemical properties in the treatment of autoimmune diseases such as nephritis and rheumatoid arthritis for centuries (Wang et al., 2012; Huang et al., 2013; Hung et al., 2013). Besides, triptolide is able to potently inhibit the growth of human cancer cells in vitro and prevents tumor growth in vivo via inhibiting cell proliferation and inducing apoptosis (Li et al., 2012; Tao et al., 2012; Chueh et al., 2013). However, triptolide possess both immunsuppressive and antifertility activities through its ability to inhibit the proliferation of both activated monocytes and spermatoocytes (Huang et al., 2012; Xiaowen et al., 2012; Zhang et al., 2012).

Artesunate (ART), is a derivative of artemisinin isolated from the traditional Chinese herb Artemisia annua L., has been approved by the Chinese government for the treatment of malaria, especially against cerebral malaria, more recently, scholars had found it has a wide range of biological activities, such as hepatoprotective, antioxidative, anti-inflammatory, anti-diabetic, antiallergic, and antibacterial effects (Ma et al., 2011; Jiang et al., 2012; Mao et al., 2012; Zhao et al., 2012). But now, a number of publications have shown that artesunate can inhibit the proliferation of cancer cells and inhibit angiogenesis in vitro and in vivo (Bachmeier et al., 2011; Berdelle et al., 2011; Zhao et al., 2011).

In order to reduce the side effects and enhance the efficacy, traditional Chinese medicine (TCM) prescriptions from synergistic combinations of different TCM herbs is alternative strategy for cancer therapy. The present study was designed to determine combined efficacy of triptolide and artesunate on pancreatic cancer cell lines in vitro and in vivo.

Materials and Methods

Cell culture

Human pancreatic cancer cell lines PANC-1, CFPAC-1 and normal pancreatic cell line HPC-Y5, were obtained

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from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained as monolayer cultures in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin and 100 U/ml of penicillin at 37 °C in a humidified atmosphere of 5% CO₂.

**Drugs and reagents**

Artesunate, provided by Guilin South Pharmaceutical Company Limited (purity > 99.0%, Guilin, Guangxi, China). Artesunate was dissolved in 1 ml of 5% sodium bicarbonate to obtain 1 mM stock solution, and then diluted in medium to different concentrations. Triptolide (purity > 99.0%, Institute of Dermatology, Chinese Academy of Medical Sciences, Nanjing, China) was prepared in dimethyl sulfoxide (DMSO) to obtain 1 μM stock solution and then was added in medium at required concentrations for a certain period of time. Methyl-thiazolyltetrazolium-bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (NY, USA). RNase A and propidium iodide (PI) were obtained from Sigma (St. Louis). TransScript First-Strand cDNA Synthesis SuperMix was purchased from TransGen Biotech (Trans, Beijing, China). GeneRuler TM 100 bp DNA Ladder and DreamTaq™ Green PCR Master Mix were purchased from Fermentas Company (Fermentas, Shenzhen, China). Primers were synthetized by Sangon Biotech (Sangon, Shanghai, China). total protein extraction kit P1250 (Applygen Technologies Inc., Beijing, China); BCA protein assay kit (Biosynthesis Biotechnology Co., Ltd., Beijing, China); Anti-Hsp20, anti-Hsp27 and anti-Hsp60 antibodies, as well as horseradish peroxidase (HRP)-conjugated goat anti-mouse and HRP-conjugated goat anti-rabbit secondary antibodies, were from Santa Cruz Biotechnology (CA, USA). Artesunate and Triptolide, Artesunate (50 – 200 μM) and Triptolide (50 nM and 100 nM). Both the floating and adherent cells were collected together for the analysis. Cells were washed with PBS and centrifuged, fixed with 70% (v/v) ice-cold methanol overnight at 4 °C. The fixed cells were collected by centrifugation, washed with PBS, and then resuspended in 100 μl of PBS containing 40 μg/ml RNase A, after being stained 5 μl Annexin V-FITC and 50 μg/ml propidium iodide, cells were placed in dark for 30 min at room temperature, then binding buffer (400 μl) was added to each tube. Analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) within 1 h of staining using Cellquest and ModFit software.

**Caspase activity determination**

The activity of caspase-3 and caspase-9 were measured with a colorimetric assay kit (BioBox, Nanjing, China) according to the manufacturer’s instruction. The assay is based on the cleavage of the chromogenic substrates, DEVD-pNA and LEHD-pNA, by caspase-3 and caspase-9, respectively. PAN-C-1 and CFPAC-1 cells (1x10⁴) were seeded into 96-well white opaque plates and a corresponding optically clear 96-well plate, and then allowed to adhere overnight. The next day, cells were treated with Artesunate and Triptolide. At the end of the incubation time, Cells were lysed in chilled lysis buffer on ice for 10 min and centrifuged for 5 min at 10,000 rpm. Caspase substrate solution containing the specific peptide substrate was then added to the supernatant and incubated for 2 h at 37 °C before measurement by microplate reader at a wavelength of 405nm.

**RNA isolation and real time RT-PCR analysis**

PANC-1 and CFPAC-1 cells were seeded in six-well plates at a density of 3x10⁴ cells and were then incubated overnight at 37 °C before treatment. After cells were treated with Artesunate and Triptolide for 48 h, total RNA was prepared using the TRIzol reagent (Tiangen, Beijing, China), according to the manufacturer’s instructions. RNA was reverse transcribed into first-strand cDNA using a kit (Tiangen, Beijing, China) following the manufacturer’s procedure. The synthesized cDNA was used as a template for polymerase chain reaction (PCR) amplification. Real-time PCR was performed using a Thermal Cycler Dice Real Time PCR System (Takara, Japan). The primers used for SYBR Green real-time RT-PCR were as follows: for Hsp20, sense primer (TCT TTG ACC AGC GCT TCG GC) and antisense primer (AGC AGC ACC GAA AAG TGG CC); for Hsp27, sense primer (CCA GAG CAG AGT CAG CCA GCAT) and antisense primer (CAG AGGT G TA CTT TGG TGA); for Hsp60, sense primer (AGT CAA GCC TCC AGG TGT TCG) and antisense primer (TGG CAT CGT TCT TGG TCA CA); and for GAPDH, sense primer (AGC CTC AAG ATC ATC AGC AAT G) and antisense primer (ATG GAC TGT GGT CAT GAG TCC TT). A dissociation curve analysis of Hsp20, Hsp27, and Hsp60 showed a single peak. PCRs were
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Results

Triptolide and artesunate enhanced inhibitory effects on pancreatic cancer cells in vitro

The viability of PANC-1 and CFPAC-1 cells treated with triptolide and artesunate was analyzed by MTT
method. As seen in Figure 1, the growth of PANC-1 and CFPAC-1 cells was significantly inhibited by triptolide (Figure 1A) and artesunate (Figure 1B) in a dose dependent manner. To evaluate the synergistic effect of triptolide and artesunate in combination for subsequent studies, the viability of PANC-1 and CFPAC-1 cells decreased more in combination compared with drug used alone, and no synergistic cytotoxicity was observed in normal cells (Figure 1C).

**Induction of apoptosis by triptolide and artesunate combination in pancreatic cancer cells**

To further explore the mechanism by which triptolide and artesunate induced cell viability loss, Annexin V/PI staining and caspase-3 and caspase-9 activity assay were used. Apoptotic cells were observed in the combination as is shown in Figure 2A, the proportion of Annexin V/PI positive cells increased in triptolide and artesunate combination. In addition, the effect of triptolide and artesunate combination on the activation of the effector caspase (caspase-3 and caspase-9) was analyzed. A significant activity increase in caspase-3 (Figure 2B) and caspase-9 (Figure 2C) activation was observed after combination treatment. These findings indicate that activation of a caspase-involved apoptotic pathway is one of the major mechanisms via which triptolide and artesunate combination exerts synergistic effect on pancreatic cancer cells.

**Hsp20 and Hsp27 down regulated by tripolide and artesunate combination**

Among the heat shock proteins, three chaperones, HSP20, HSP27 and Hsp60 were reported as important target in anti-tumor therapy (Guo et al., 2008; Edwards et al., 2011; Hjerpe et al., 2013). We were interested to know how they keep their function in triptolide and artesunate combination. As indicated in Figure 3A and Figure 3B, the mRNA level of HSP20 and HSP27 decreased in both PANC-1 and CFPAC-1 cells, however, triptolide and artesunate combination had no effect on Hsp60 (C). Moreover, PANC-1 cells were incubated with triptolide and artesunate alone or combination for 24 hours, effect of TPL combined with artesunate on the protein levels of HSP20 and HSP27 (D) and Hsp60 (E) was analyzed by Western blot analysis. Hsp20 and Hsp27 downregulated by triptolide and artesunate combination and Hsp60 was unchanged in PANC-1 and CFPAC-1 cells (Figure 3C). From the Western blot analysis, HSP20 and HSP27 expression decreased (Figure 3D) but HSP60 and is consistent with their mRNA expression level analyzed by qRT-PCR. Therefore, our results indicate that triptolide...
cooperates with artesunate to induce the decline of HSP20 and HSP27 expression via a pharmacological manner, which may be the crucial mechanism underlying the synergic apoptosis on pancreatic cancer cells.

Antitumor effects of triptolide in combination with artesunate: in vivo assay

The antitumor effect of triptolide and artesunate in combination was analyzed in a xenograft tumor model (pancreatic cancer cells bearing mice). After implantation for 7 days, the tumor xenografts reached size of 120 ± 12 mm3. We chose 45 mice with tumor xenografts of around 100 mm3 in size and randomly divided them into four groups (with 5 mice per group). There were no statistical differences among the sizes of all the groups. Thereafter, the mice were given different treatments. No significant change in body weight was observed in the mice treated with triptolide alone, artesunate alone, or combined triptolide and artesunate (Figure 4A). The tumor weight treated by drugs were decreased compared with the saline group, while degree of tumor decreased more in combination group compared with triptolide and artesunate used separately (Figure 4B). These results show that triptolide combined with artesunate have synergic antitumour effect.

Discussion

Pancreatic cancer is highly resistant to current chemotherapy agents. Recently, tumor therapy by traditional Chinese herb is becoming more and more attractive (He et al., 2008; Youns et al., 2009; Chen et al., 2010). We therefore attempted to examine the effects of triptolide and artesunate on pancreatic cancer.

In this study, we shown that triptolide combined with artesunate acts in synergy to inhibit pancreatic cancer cell line growth and induce its apoptosis. HSP20, HSP27 and HSP60 play an important role in carcinogenesis and tumor chemotherapy (Matsushima-Nishiwaki et al., 2007; Noda et al., 2007; Matsushima-Nishiwaki et al., 2008; Cappello et al., 2013). It’s over expression is associated to antiapoptosis property and chemotherapy resistance in a variety of tumors including prostate, breast, gastric, and colon cancers. Newly reports have shown that these heat shock proteins may be act as a target for anti-tumor therapy (Li et al., 2008; Du et al., 2010). In the present study, we found that HSP20 and HSP27 declined during combination. That means that the synergetic inhibition induced by triptolide and artesunate combination was more likely due to the proliferation related pathway triggered by HSP20 and HSP27 but HSP60. Moreover, the synergetic effect of triptolide and artesunate in vitro was verified in xenografts from PANC-1 and CFPAC-1 cells. In vivo, tumors with combination therapy grow slowly, and the tumor weights were much lighter than the drugs used separately.

In summary, the present study has demonstrated that growth inhibition by triptolide and artesunate combination on pancreatic cancer cells resulted from the induction of apoptosis in vitro, and this cooperation effectiveness is also exert in the pancreatic cancer cell bearing xenograft model in vivo. Our findings suggest that this approach effectively suppresses the growth of pancreatic cancer in vivo and in vitro.

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References


