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Effects and Mechanisms of Trifluridine alone or in Combination with Cryptotanshinone in Inhibiting Malignant Biological Behavior of Gastric Cancer

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Abstract

Background: Gastric Cancer (GC) is the fourth most common malignant tumor worldwide. This study aimed to investigate the effect of a combination of Cryptotanshinone (CTS) and Trifluorothymidine (FTD) on GC.

Methods: The effect of the combined or separate use of FTD and CTS on HGC-27 and AGS cells was detected using the CCK8 assay. The combined index of FTD and CTS was calculated using Compusyn software. Additionally, we applied flow cytometry to study the cell cycle and apoptosis and investigated the amount of FTD incorporated into DNA by immunofluorescence assay. Protein expression was monitored by western blotting (WB). Furthermore, the effect of TAS-102 in combination with CTS was studied in a xenograft nude mouse model.

Results: FTD and CTS inhibited the proliferation of GC cells in a dose-dependent manner. The combination of FTD and CTS showed synergistic anticancer effects in HGC-27 and AGS cells. Our mechanistic studies indicate that FTD blocks HGC-27 cells at the G2/M phase, CTS blocks HGC-27 cells at the G1/G0 phase, while FTD combined with CTS mainly blocks HGC-27 cells at the G2 phase. The combination of FTD and CTS significantly increases apoptosis. CTS increased the incorporation of FTD into the DNA. FTD activated STAT3 (Signal Transducer and Activator of Transcription 3) phosphorylation, whereas CTS downregulated p-STAT3. Interestingly, the combination of CTS and FTD reduced the STAT3 phosphorylation induced by FTD. In vivo, the combination of TAS-102 with CTS was significantly more potent than TAS-102 alone in tumor growth inhibition.

Conclusions: FTD combined with CTS has a synergistic anti-gastric cancer effect in vitro and in vivo and is a promising treatment option for advanced GC.

Keywords: Trifluorothymidine; Cryptotanshinone; Gastric cancer; Synergy; STAT3

Introduction

Gastric cancer is a malignant tumor with high morbidity and mortality in China [1]. Currently, there are no specific or highly effective drugs that can prevent the development of gastric cancer. Currently, chemotherapy such as taxus, platinum, and pentafluoride is the main method of multidisciplinary comprehensive treatment for advanced gastric cancer. The chemotherapy regimen mainly includes taxus, platinum, and pentafluoride, but its efficacy is poor and there are many adverse reactions after chemotherapy for gastric cancer [2].

The chemical structure of Cryptotanshinone (CST) is C19H20O3 with a relative molecular weight of 296.35. Cryptotanshinone is extracted from the roots and rhizomes of Salvia miltiorrhiza and mainly exists in Salvia and Sage. Many studies have shown that CTS has a wide range of pharmacological properties, including anti-tumor anti-inflammatory, neuroprotective, cardiovascular protective, anti-fibrosis, and anti-metabolic effects [3]. CTS have also received increasing attention in recent years owing to its anti-cancer activity. Previous experimental results from our group have shown that CTS can effectively activate the apoptotic pathway and block gastric cancer cells in the G0/G1 phase. CTS, a natural inhibitor of STAT3 (Signal Transducer and Activator of Transcription 3), can inhibit cell proliferation by inhibiting the downstream protein of STAT3. Studies have found that CTS can effectively improve the efficacy of doxorubicin, paclitaxel, arsenic trioxide, and other anticancer drugs through a process mediated by the STAT3 signaling pathway.

TAS-102 is a promising anti-tumor agent that can be used in combination with other chemotherapeutic agents. TAS-102 is a combination of a novel oral nucleoside analog trifluorouracil (FTD) and thymine phosphorylase inhibitor tipilasi hydrochloride (TPI) in a molar ratio of 1:0.5. FTD is the active cytotoxic component of TAS-102, and TPI plays a role in prevent rapid degradation and inactivation of FTD by thymine phosphorylase in vivo. FTD enters the body via nucleoside transporters and is sequentially phosphorylated by thymidylate kinase. Its monophosphate-phosphate form (FTD-MP) can temporarily inhibit thymic acid synthase (TS) and its triphosphate form (FTD-TP) can be inserted into DNA strands [4]. TS inhibition is the main mechanism of action of classical fluoropyridines such as 5-FU. Although the inhibition of TS by FTD-MP may partially explain the anti-tumor effect of FTD, studies have shown that the incorporation of FTD-TP into the DNA chain, resulting in DNA damage, is the

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main mechanism of FTD. FTD is one of the few chemotherapeutic agents that can promote STAT3 phosphorylation for the treatment of advanced tumors.

The purpose of this study was to evaluate the efficacy and potential mechanism of FTD alone or in combination with CTS for the treatment of gastric cancer in vitro and in vivo. We studied the effect of the combination of FTD and CTS by investigating its influence on cell proliferation pathways through the cell cycle, apoptosis, and the incorporation of DNA strands.

Materials and Methods

Cell culture and reagents

The human GC cell lines HGC-27 and AGS were purchased from Procell Life Science and Technology Ltd. GC cells were cultured DMEM (HGC-27) and RPMI 1640 (AGS), respectively, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution at a 37 cell incubator with 5% CO_2.

DMEM and RPMI 1640 medium was bought from HyClone (USA). TAS-102, FTD, and CTS were obtained from TargetMol (China), and their purity was greater than 98%. Cell Counting Kit (CCK)−8 and cell cycle analysis kit were obtained from Beyotime (China), and the Annexin V-FITC/PI double staining cell apoptosis detection kit was purchased from KeyGEN BioTHCE (China) [5]. The primary antibodies included bromodeoxyuridine (BrdU), Cyclin B1, Cyclin D1, Cleavd-Caspase 3, Cleavd-Caspase 9, Bcl-2, Survivin, p-ERK (Thr202/Tyr204), ERK, p-AKT (Ser473), AKT, p-STAT3 (Tyr705), STAT3 (Cell Signaling Technology), p-JAK2 (Tyr221), JAK2, GAPDH, β-actin (Affinity), MPM-2(Abcom), IL-6, and Ki-67(Wanleibio). The secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse IgG and Cy3 labeled goat anti-mouse IgG, were from ZSGB-Bio (China) and Abcalonal (China), respectively.

Cell proliferation assay

The cell proliferation capacity of FTD, CTS, and their combination was determined by the CCK-8 assay according to the manufacturer's protocol. First, HGC-27 and AGS cells at the exponential growth stage were seeded in 96-well plates at a density of 2×10^3 cells/well. Then, different concentration gradients of DMSO, (0.125, 0.25, 0.5, 1, 2, 4, 8, 16μM) FTD, (1, 2, 4, 8μM) CTS, and thier combination were added to the culture media after cells adhered to the wall, and the cells were further cultured for another 48 hours In the environment treated with different concentrations of drugs. Finally, Added 10ul of CCK8 reagent to each well and underwent incubation as kept away from light at 37°C for 1 to 2 h [6]. The Optical Density (OD) at a wavelength of 450 nm was measured using a universal microplate reader (Bio-tek). Each assay was performed two or three times.

Wound-Healing assay and transwell invasion assay

Five uniform horizontal lines were drawn on the back of the 6-well plate using a marker pen. After treatment with 8uM CTS or 0.5μM FTD alone and in combination for 24h, the spear was used to scratch along the ruler perpendicular to the horizontal line, then washed three times with PBS (phosphate-buffered saline) and replaced with serum-free medium the next day. Images were obtained at 0, 12 and 24h using an inverted microscope (Zeiss, Germany). Magnification, x100 [7]. Invasion assays were performed using 24-well Transwell plates coated with Matrigel. After treatment with the drug at the above concentration for 24h, the cells were collected and prepared into single-cell suspensions. HGC-27 cells (1×10^5 cells) were plated in the upper transwell chamber of DMEM without FBS. 600 μL DMEM medium supplemented with 10% FBS was added to the lower chambers. The cells were cultured in an 37°C incubator for 24h. The cells travelled through the matrix gel to the lower surface and were then fixed and stained with crystal violet. Images were captured using an inverted microscope.

Cell cycle analysis

HGC-27 cells (1×10^5cells/well) were seed in 6-well plates and starved overnight. And cells were treated with constant concentration of CTS (8uM) and different dose of FTD (0.25 and 0.5μM) alone or their combination (CTS 8uM + FTD 0.25 or 0.5uM) for 72h. Cells were harvested and converted into a single-cell suspension, washed with cold phosphate-buffered saline (PBS) three times, and then fixed with 70% cold ethanol at 4 °C overnight. The cells were centrifuged and resuspended in PBS. RNaseA and propidiumiodide (PI) were added and incubated for 30min at 37°C. Flow cytometric analysis was carried out for 30 min using CytoFLEX flow cytometry (Beckman, USA), and frequency results for different stages of the cell cycle were determined using FlowJo software (v. 7.6.1). The DMSO group was used as a control. Each experiment was performed two or three times.

Cell apoptosis analysis

Apoptotic cells were analyzed using an Annexin V-FITC/PI double staining cell apoptosis detection kit. HGC-27 cells were seeded at a density of 1×10^5 cells/well in 6-well culture plates. The cells were treated with the indicated concentrations of FTD and/or CTS for 48h until grew with adherent. The cells were digested with trypsin without EDTA, collected, and washed twice with PBS. Annexin V-FITC (5 μL) and propidium iodide (PI) (5 μL) were added to the cell suspension. After mixing, the cells were maintained at room temperature and protected from light for 15 min [8]. A CytoFLEX flow cytometer (Beckman, USA) was used for the analysis. The percentage of apoptotic cells was determined using FlowJo software.

Immunofluorescence assay

HGC-27 cells (1×10^5cells) were evenly seeded on coverslips. The cells were treated with drugs for 24h, fixed with 4% formaldehyde for 15 min, and washed with PBS three times. Next, 0.1% TritonX-100 to completely cover the cells, which were then incubated at room temperature for 30 min and washed three times with PBS. The cells were blocked in 5% goat serum at room temperature for 1h. BrdU primary antibody was diluted with PBS at 1:500 and incubated overnight at 4°C. The next day, after washing three times with PBS, the cells were incubated with a Cy3-conjugated secondary antibody (1:100 dilutions) for 1h at room temperature in the dark. The cell nuclei were stained with DAPI for 10 min. The cells were washed as before, and the plate was sealed with an anti-fluorescence quenching agent. Finally, the images were observed using an inverted fluorescence microscope (×400).

Western blotting

Western blotting was performed as previously described [9]. Briefly, after drug treatment for 24 h, total protein was extracted using RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using a BCA protein assay kit according to the manufacturer's protocol. Equal amounts of total protein (40μg) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. The Membranes were blocked with 5% skim milk at 37°C for 2h. The protein we are interest in on the membrane was cut horizontally along the position suggested by prestain marker, and

subsequently incubated with the specific primary antibody at 4°C overnight. GAPDH or β-actin served as internal controls. The next day, the membranes were washed three times with TBST (Tris-buffered saline and 0.1% Tween-20) and incubated at room temperature for 1h with the corresponding secondary antibody. Each band was examined by adding chemiluminescence (Thermo Scientific) to the membrane and the results were captured using a gel imaging system (Tanon 5200).

Hematoxylin–eosin (HE) staining

After treatment, parts of the liver and kidney tissues of the nude mice were collected, fixed with formaldehyde, dehydrated, and then embedded in paraffin. After cooling, the tissues were cut into 5μm thick sections. The sections were dewaxed with xylene and hydrated with gradient ethanol. The sections were stained with hematoxylin for 5 min, eosin for 3 min, and then washed with running water for 10 min. The slices were dehydrated and sealed with a neutral resin. Finally, a positive light microscope (×200) was used to observe images on the slides.

Immunohistochemistry

The tumor, liver, and kidney tissues of nude mice were fixed, embedded in paraffin, and sectioned into 5μm thick slices. The sections were dewaxed with xylene and hydrated with gradient ethanol. Antigen retrieval was performed using citrate antigen retrieval solution at 100°C for 10min. The immunohistochemical pen was drawn in a circle around the tissue and incubated at room temperature with 3% H_2O_2 for 15 min to eliminate endogenous peroxidase activity, and then soaked in PBS for 5 min and repeated three times. After incubation with goat serum at room temperature for 1h, the primary antibody was diluted with PBS at 1:100, and then dropped until the tissue was completely covered [10]. The antibody was stored in a wet box at 4°C overnight. On The next day, soaked again in PBS for three times (5 min per wash), the HRP-conjugated secondary antibody was dropped to completely cover the tissue, and was incubated in a wet box at 37°C for 60min, followed by soaking in PBS for 5min, and repeated for three times. The DAB chromogenic agent was applied to the section and placed in water to stop the reaction when the color became dark. After staining the cells with hematoxylin, the sections were dehydrated and sealed with a neutral resin. Images were observed under a microscope (×400).

Nude mice xenograft tumor model of human HGC-27

Female BALB/c nude mice, weighing 18–20 g and aged 5 weeks, were purchased from Wanlei Biotechnology Co., Ltd. All animal experiments were performed in accordance with the Committee of Animal Experimentation and Ethics Committee of the Anhui Medical University (Anhui, China). These nude mice were kept in a specific pathogen free (SPF) environment with 12h light/dark cycle, 22±1°C temperature, 45%-55% humidity, and free access to food and water. After adaptive feeding for one week, 100μl cell suspension of HGC-27 cell suspension was injected into the axilla of nude mice at a density of 4×10^7 cells/mL. All nude mice were randomly divided into three groups: control group (DMSO), TAS-102 treatment group (TAS-102), and combined treatment group (TAS-102+CTS). Each group was assigned to three nude mice [11]. The control group was injected with an equal dose of DMSO every two days. The TAS-102 treatment group was orally administered TAS-102 twice daily (each time 75 mg/kg), with an interval of 6h. In the combined treatment group, TAS-102 was administered orally twice daily at 6h interval, and CTS was injected intraperitoneally every two days (10 mg/kg). Tumor volume (V) was measured every 3 days and tumor volume were calculated according to the formula V=0.5×L×S2. After 14 days of drug treatment, nude mice were sacrificed routinely, and their kidney, liver, and tumor tissues were collected for follow-up experiments.

Statistical analysis

The combination index (CI) determined by the Chou-Talalay method can be used as an indicator of drug interactions. A CI < 1 was classified as synergy, CI = 1 was classified as additive, and CI > 1 was interpreted as antagonism. The CI value was calculated using CompuSyn software (V. 2.0). In addition, fa represents the fraction of cells affected and the dose-reduction index (DRI) is the fold-decrease of each single agent when two or three drugs are used in combination. GraphPad Prism software (version 6.0) was used to calculate the IC_{50} values and plot statistical graphs. All data are presented as mean ± SD. The differences between the two groups were compared using the Student's t-test, and differences between multiple groups were compared using the analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

Result

The combination of FTD and CTS has a synergistic anticancer effect on gastric cancer cells

Cryptotanshinone (CTS), a natural product extracted from Salvia miltiorrhiza, has been discovered as an anti-cancer drug. To investigate the antiproliferative activity of FTD and CTS, human gastric cancer cells were treated with different concentrations of each drug for 72h. FTD and CTS significantly inhibited the proliferation of these two gastric cancer cell lines in a dose-dependent manner. The IC_{50} values of cryptotanshinone for HGC-27 and AGS cells were 10.68 μmol/L and 5.43 μmol/L, and the IC_{50} values of FTD for them were 0.87 μmol/L and 2.36 μmol/L, respectively [5]. A drug concentration close to IC_{50} was selected as the subsequent experimental concentration. In addition, to further analyze the effect of the combination of the two drugs on gastric cancer cells, we also analyzed the effects of 2, 4, and 8 μmol/L CTS combined with different concentrations of FTD on HGC-27 cells and 1, 2, and 4 μmol/L CTS combined with different concentrations of FTD on AGS cells. Our results revealed that the combination of FTD and CTS resulted in a pronounced decrease compared with alone in cell viability. The combination index (CI) of FTD and CTS in gastric cancer cells was assessed using the CompuSyn software. When the fraction of cells affected (Fa) was 50%, the concentration of FTD alone was more than three times that of the combination in the HGC-27 cells. FTD and CTS were synergistic in HGC-27 cells with CI values ranging from 0.566 to 0.878 for Fa=0.1~0.75, as indicated and the Fa-CI plot. Similarly, when the Fa of AGS cells was less than 95%, the CI values of FTD and CTS were less than 1, indicating that FTD and CTS also had a synergistic effect in AGS cells [12]. In particular, HGC-27 cells were highly sensitive to FTD. Hence, HGC-27 cells were selected for further studies. We also explored the biological role of FTD and its combination in HGC-27 cells. Compared with the control group, both FTD and CTS inhibited the migration and invasion abilities of HGC-27 cells, and more importantly, the inhibitory effect was more obvious when FTD was combined with CTS. Overall, our results indicated that FTD and CST have synergistic anti-gastric cancer effects *In vitro*.

FTD and its combination with CTS cause cell cycle arrest

Since FTD combined with CTS can significantly inhibit the proliferation of gastric cancer cells, we conducted a cell cycle analysis to explore the mechanism of their synergistic anticancer effects. Firstly, HGC-27 cells were treated with different concentrations of FTD for 72h, Compared with the control group we found that the cells were

arrested in the G2/M phase in a dose-dependent manner (P<0.05), and treatment with 0.5μmol/L FTD increased the proportion of HGC-27 cells in the G2/M phase from 18.52% to 49.82%. In addition, 8μmol/L CTS could block HGC-27 cells in the G0/G1 phase. In the combination treatment group, the proportion of HGC-27 cells in the G2/M phase was significantly higher than that in the control group but slightly lower than that in the FTD alone treatment group. Furthermore, to determine the molecular mechanism of FTD and its combination with CTS in HGC-27 cells, we investigated the expression of cell cycle-associated protein markers by western blotting. Cyclin D1 protein expression levels were downregulated when treated with CTS alone, and Cyclin B1 protein expression levels were dose-dependently increased when treated with CTS alone, which was consistent with the results of cell cycle analysis [13]. In the combination treatment group, the expression of Cyclin D1 protein showed no significant change compared with the control group, while Cyclin B1 protein was still upregulated. Finally, we analyzed the fraction of HGC-27 cells in the mitosis stage by measuring the expression of the polyclonal antibody MPM-2 protein, which was found to be levels of MPM-2 protein in the combination treatment group lower than in the other groups. This demonstrates that FTD and its combination with CTS treatment of HGC-27 cells results in G2/M phase arrest, which is mainly in the G2 phase rather than in the mitotic phase.

FTD treatment or in combination with CTS induces cell apoptosis

To investigate whether FTD and its combination with CTS could induce apoptosis, HGC-27 cells were treated with different concentrations of FTD and CTS, alone or in combination for 72h. As shown in Fig 3A and Fig 3B, the proportion of apoptotic cells was significantly rise up to 27.04% at 0.5uM FTD (P<0.01) and 8.12% at 8μM CTS in HGC-27 cells. In the combination of 0.25μM FTD+8μM CTS and 0.5μM FTD+8μM CTS treated group, the percentage of the apoptotic HGC-27 cells significantly increased 15.09% and 19.82%, respectively, compared with that in 0.25μM FTD and 0.5μM FTD treated groups (P<0.05) [4]. Because combination treatment induced apoptosis in HGC-27 cells, we measured the levels of Cleavd-Caspase 9 and Cleavd-Caspase 3. The expression levels of the anti-apoptotic proteins Bcl-2 and Survivin in each treatment group were also measured by western blotting. As showed in Fig3C, the expression of Cleavd-Caspase 3 and Cleavd-Caspase 9 was significantly upregulated in a dose-dependent manner after exposure to FTD and its combination treatment, which decreased the expression of Bcl-2 and Survivin. The level of Cleavd-Caspase 9 in the combination group was higher than that in the FTD alone group. Taken together, our results suggest that combined treatment with FTD and CTS could induce more apoptosis in HGC-27 cells than in any other group.

CTS increased the amount of FTD incorporation into genomic DNA

Since FTD can be identified by BrdU antibody, we analyzed the amount of FTD incorporated into the DNA strand using immunofluorescence assay. Our results showed that FTD incorporation into HGC-27 cell DNA was concentration dependent. In the combination of 0.25μM FTD+8μM CTS. The level of FTD incorporation into DNA was significantly higher than that by 0.25μM FTD only group. Further increases in FTD incorporation were observed when FTD was combined with CTS, which reduced DNA synthesis in gastric cancer cells.

CTS blocked the FTD-induced phosphorylation of STAT3 via affecting its upstream signaling pathway

As is known to all, constant activated STAT3 increases tumor cell proliferation and inhibits anti-tumor immunity. Our immunoassay results showed that FTD treatment increased the phosphorylation of STAT3 at Tyr-705 in a dose-dependent manner, but had no significant effect on the total STAT3 protein. In addition, 8 μM CTS attenuated the levels of phosphorylated STAT3. Compared to FTD treatment, FTD in combination with CTS significantly reduced STAT3 phosphorylation induced by FTD. To further elucidate the potential molecular mechanism of this result, we examined the main STAT3 upstream signaling cascade pathways, including the JAK/STAT3, MEK/ERK, and PI3K/AKT/mTOR pathways, and the critical protein expression status, especially alterations at the phosphorylation level [14]. Our results suggested that P-AKT (Ser473), P-ERK (Thr202/Tyr204), and P-JAK (Tyr931) were reduced in the FTD combined with CTS-treated HGC-27 cells compared with the FTD alone treatment, while the total protein expression levels of AKT, ERK, and JAK were hardly affected. These data indicated that CTS could reduce FTD-induced STAT3 phosphorylation by repressing the activation of STAT3 upstream signaling pathway proteins.

FTD in combination with CTS can significantly enhance the anticancer ability in nude mice subcutaneous HGC-27 xenograft tumor model

To further elucidate the anticancer effect of TAS-102 combined with CTS *in vivo*, we established a subcutaneous HGC-27 xenograft tumor model. Nine nude mice were randomly divided into three groups: DMSO, TAS-102, and TAS-102+CTS groups. As shown in Fig 6B, compared with the DMSO group, TAS-102 treatment alone inhibited tumor growth by 13.45%, while the tumor growth inhibition rate of the combination reached 52.65%. Additionally, we examined whether TAS-102 and CTS treatment had side effects on the liver and kidney in nude mice at effective doses using hematoxylin-eosin staining [11]. Our results show that No inflammatory response was observed in the liver and kidneys of the treated nude mice. Additionally, immunohistochemical experiments showed that there was no significant difference in IL-6 levels in the liver and kidney of nude mice in each treatment group compared to the control group. In addition, we detected the expression level of FTD in the xenograft tumor tissues by BrdU antibody, which was consistent with the results of immunofluorescence we had done before. There was a significant decrease in the expression level of Ki-67 in the TAS-102+CTS group compared with that in the TAS-102 and DMSO groups. Compared with the DMSO group, p-STAT3 expression increased in the TAS-102 group, but returned to a lower level after combined CTS treatment. Therefore, CTS significantly enhanced the anticancer activity of TAS-102 *In vivo*.

Discussion

To date, early diagnosis of gastric cancer remains difficult, and gastric cancer is often at an advanced stage when clinical symptoms appear. Although most patients with gastric cancer can be treated surgically, the diagnosis is poor. The five-year survival rate after surgery is only 20%-30%. As a newly discovered anti-tumor drug, FTD has attracted considerable attention owing to its unique anti-tumor mechanism and strong anti-cancer ability. FTD has been recommended as third-line chemotherapy for HER2-negative patients with advanced gastric cancer according to the latest 6th Edition of Japanese Gastric Cancer Treatment Guidelines. Traditional chemotherapeutic drugs are associated with serious adverse reactions and high drug resistance

rates. However, Traditional Chinese medicine has great potential in the treatment of advanced gastric cancer owing to its advantages of improving patients' quality of life, low toxicity, side reactions, and sensitization chemotherapy. Therefore, understanding the effect and mechanism of the combination of FTD and CTS in gastric cancer treatment is of great importance to doctors. Our results are the first to reveal the *in vitro* and *in vivo* combinational effects of FTD and CTS in gastric cancer.

Chemotherapy is the primary method of comprehensive multidisciplinary treatment for advanced gastric cancer. The recommended first-line chemotherapy regimen for gastric cancer includes oxaliplatin combined with fluorouracil (FOLFOX or XELOX), docetaxel/paclitaxel combined with cisplatin (TP), and irinotecan combined with fluorouracil (FOLFIRI), but its efficacy has not been satisfactory. As a novel fluorouracil anti-tumor agent, TAS-102 was first approved in Japan in 2014 for the treatment of unresectable advanced or recurrent colorectal cancer [15]. The study of TAS-102 in patients with gastric cancer began with a phase 3 trial in a large sample (n=507) of patients with metastatic or advanced gastric cancer in Eastern and Western countries who had received at least two previous chemotherapy treatments. The results showed a 31% reduction in the risk of death in the TAS-102 group compared with that in the placebo group. Based on this study, TAS-102 was recently approved by the Food and Drug Administration (FDA) for gastric or gastroesophageal junction adenocarcinoma in patients who had previously received at least two chemotherapy regimens, including fluorouracil, platinum compounds, taxane, and irinotecan [11]. Here, our results demonstrate that FTD and CTS combined treatment of gastric cancer cells had significantly stronger inhibition of proliferation than each single drug treatment group. More importantly, the two drugs showed synergistic anticancer effects at appropriate concentrations. Previous studies have shown that TAS-102 may enhance the efficacy of combination therapy with the chemotherapy agents irinotecan, oxaliplatin, and the targeted chemotherapeutic agent bevacizumab.

We further analyzed the potential mechanism of the synergistic effect of FTD and CTS on the cell cycle and apoptosis. Compared with the control group, CTS alone blocked HGC-27 cells in the G1 phase and significantly reduced Cyclin D1 levels, which was consistent with our previous findings. FTD blocks cells in G2 phase and upregulates Cyclin B1 expression. After combining FTD and CTS, we found that the cells were mainly block HGC-27 cells at the G2 phase, and the expression level of mitotic phosphoprotein monoclonal antibody(MPM)-2 protein decreased sharply, indicating that most of the cells were blocked in the intercellular phase and did not enter the mitotic phase. In general, mAbs recognize only one or a few specific proteins. However, MPM-2 recognized more than 50 proteins. Mitogen-specific MPM-2 antigens have conserved phosphorylation epitopes present in a wide range of species. Phosphorylation of these epitopes occurs during mitotic induction and is concentrated in mitotic subcellular structures, such as centrosomes, centromeres, spindle fibers, and chromosome axes [10]. FTD combined with CTS increased the expression of pro-apoptotic proteins Cleavd-Caspase 3 and Cleavd-Caspase 9 and inhibited the expression of anti-apoptotic proteins Bcl-2 and Survivin, thereby promoting the apoptosis of gastric cancer cells. FTD works by incorporating DNA strands into tumor cells to inhibit DNA replication. In this study, immunofluorescence was used to intuitively observe that CTS could promote FTD incorporation into the DNA chain. Similarly, Baba et al. found that 5-FU could promote FTD incorporation into the DNA chain [5].

STAT3 is involved in cell cycle(cMyc and Cyclin D1), anti-apoptotic (Bcl-XL, Bcl-2 and Survivin), angiogenesis(VEGF and IL-8), and invasion and migration(MMP-2 and MMP-9) regulation gene expression [1]. Additionally, the JAK/STAT pathway is a key signaling pathway for the activation of the tumor immune system. To activate the JAK/STAT pathway, cytokines, such as IL-6, bind to their receptors to activate JAK trans-phosphorylation, which then recruits and catalyzes STAT phosphorylation. Phosphorylated STAT forms a dimer and subsequently moves into the nucleus to initiate the transcription of target genes [6]. Previous studies have shown that the JAK2/STAT3 signaling pathway is involved in the occurrence and development of various solid tumors, such as colorectal cancer, hepatocellular carcinoma, and ovarian cancer, and is over-activated in tumor cells [15]. The JAK2/STAT3 signaling pathway and targeted therapy for STAT3 have attracted wide attention in recent years. In this study, we investigated HGC-27 cells treated with FTD alone and found that the phosphorylation of STAT3 was promoted when the total STAT3 protein expression was unchanged, whereas the expression of P-STAT3 was downregulated by CTS alone. CTS combined with FTD reduced the phosphorylation level of STAT3 induced by FTD compared with FTD alone. In addition, we studied the expression levels of ERK, AKT, and JAK2, the main upstream signaling pathway proteins, to determine the potential mechanism of the changes in p-STAT3 expression caused by CTS combined with FTD treatment. Extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPK) family, is an upstream signal of STAT3. Its signaling pathway is the core of the signaling network involved in regulating cell growth, development, and division [7].

Our results on nude mice xenograft tumors showed that FTD and CTS combined therapy could more significantly inhibit the growth and malignancy of gastric cancer while exhibiting minimal liver and kidney toxic side effects that are commonly caused by many chemotherapy drugs. There were also some limitations in this study. The synergistic anticancer mechanism of FTD and CTS in AGS cells has not been further elucidated. In addition, the specific mechanism by which CTS promotes FTD insertion into the DNA strands was not investigated in this study.

Conclusion

In conclusion, the combination of FTD and CTS can play an anticancer role through the cell cycle and cell apoptosis, and can influence cell proliferation pathways by increasing the incorporation of DNA chains, which are closely related to the occurrence and development of gastric cancer. The combination of FTD and CTS can enhance the anti-tumor effect and reduce the toxicity and side effects of chemotherapy, which is expected to have great potential in the treatment of advanced gastric cancer.

Ethics declarations

The present study (LLSC20200185) was reviewed and approved by the Animal Care and Use Committee of the Anhui Medical University. I confirm that all experiments were performed in accordance with the relevant guidelines and regulations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

Not applicable.

Contributions

PQL, LXZ, and ZMC performed the experiments, conducted the statistical analysis, and drafted the manuscript. GW assisted in performing the experiments. HZ and SCY discussed the results and helped revise the manuscript. AMX, WXH, and ZJW designed the main study and critically revised the manuscript. All authors have read and approved the final manuscript.

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