Life Sciences 144 (2016) 138–147

Contents lists available at ScienceDirect

Life Sciences

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

Luteolin synergizes the antitumor effects of 5-fluorouracil against human hepatocellular carcinoma cells through apoptosis induction and metabolism

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ARTICLE INFO

Article history:
Received 18 July 2015
Received in revised form 10 October 2015
Accepted 1 December 2015
Available online 2 December 2015

Keywords:
5-Fluorouracil
Luteolin
Synergistic effect
Apoptosis
Hepatocellular carcinoma

ABSTRACT

Aims: Some compounds derived from Chinese medicine have demonstrated great prospective roles in sensitization to chemotherapy. This study aimed to investigate the combination of luteolin and 5-fluorouracil on proliferations of hepatocellular carcinoma cells and the potential mechanisms.
Main methods: The antitumor effects of luteolin, 5-fluorouracil, and their combinations were detected by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate assay, and isobole method was used to evaluate drug combinations. CellTiter-Blue and Caspase-Glo 3/7 assay were used for assessment of cell viability and apoptosis after treatment with luteolin, 5-fluorouracil and their combinations. Cell cycle distributions and apoptosis were detected by PI staining, Hoechst 33342 staining and FITC-Annexin V/PI staining. Bcl-2, bax, p53 and PARP expressions were determined by Western blot. Furthermore, mRNA levels of 5-fluorouracil metabolism related enzymes were detected by RT-PCR.
Key findings: Drug combination study showed that luteolin could synergize the antitumor effects of 5-fluorouracil at different dose ratios (luteolin: 5-fluorouracil = 10:1, 20:1, 40:1) against HepG2 and Bel7402 cells. Cell viability and cell apoptosis analysis showed that the synergistic growth inhibition caused by combined luteolin and 5-fluorouracil was closely related to apoptosis. Further mechanism studies showed that the synergistic effects of drug combinations were related with enhanced bax/bcl-2 ratios and p53 expressions, and induced PARP cleavage. Also, combined luteolin and 5-fluorouracil could significantly decrease the dihydropyrimidine dehydrogenase.
Significance: These results showed that luteolin could synergize the antitumor effects of 5-fluorouracil on HepG2 and Bel7402 cells, which might be related with apoptosis and regulation of 5-fluorouracil metabolism.

1. Introduction

Traditional chemotherapy is still an important treatment for many malignancies although many patients experience unacceptable toxicity. Various efforts have been made to optimize the chemotherapeutic efficacy and minimize toxicity [1]. Combination therapy, which could kill cancer cells more effectively by targeting multiple molecules and pathways simultaneously, is extensively studied [2]. The main aims of these combination studies are to achieve synergistic therapeutic effect using lower drug doses and to minimize or delay the emergence of resistance.

Some flavonoids alone, or in combination with other agents, have been shown to possess antitumor effects both *in vitro* [3–6] and *in vivo* [7] with little toxicity to normal cells, such as epithelial cells, peripheral blood and myeloid cells [8,9]. Furthermore, some flavonoids showed obvious antiproliferative activity against many multidrug-resistant cancer cell lines [10,11]. Thus, finding effective plant-derived flavonoids with high therapeutic effects and low side effects that can be used in combination with existing chemotherapeutic agents may provide an important way in cancer treatment.

Luteolin is a naturally occurring flavonoid present in a variety of edible plants [12]. It has been well reported that luteolin could induce cell cycle arrest and or apoptosis in many human cancer cells [13–16]. Our previous study showed that luteolin was one of the most important components in extract of *Scutellaria barbata* D. Don., which could synergize the antitumor effects of low dose 5-fluorouracil [17]. Recent studies showed that luteolin could sensitize tumor necrosis factor (TNF) or TNF-related apoptosis-inducing ligand induced apoptotic cell death [18,19], suggesting its potential value in cancer combination therapy.

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http://dx.doi.org/10.1016/j.lfs.2015.12.002
0024-3205/

The low selectivity and unacceptable toxicity is a significant limitation to the clinical use of 5-fluorouracil (5-FU), especially in the hepatocellular carcinoma chemotherapy. Finding new anticancer drugs that can be used in combination with low dose 5-FU to achieve high therapeutic effect may provide an important way in hepatocellular carcinoma treatment. In this study, we examined the effects of luteolin combined with low dose 5-FU against hepatocellular carcinoma cells and then investigated the mechanisms possibly involved.

2. Materials and methods

2.1. Materials and reagents

Luteolin with the purity above 95% was provided by National Center for Pharmaceutical Screening, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China; 5-FU injection (250 mg/10 ml) was purchased from Xudonghaipu parmaceutical company, Shanghai, China; RPMI1640 medium, DMEM medium, fetal bovine serum (FBS), Trypsin-EDTA were purchased from Gibco, Grand Island, NY, USA; Regents for Western blot were purchased from Bio-Rad, USA; Bcl-2, bax, p53, PARP and GAPDH antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from cell signaling (Beverly, MA, USA); Annexin V-FITC/PI apoptosis detection kit was obtained from Invitrogen, Carlsbad, CA, USA; APOPCYTO Caspase-3 Colorimetric Assay Kit was obtained from MBL, Nagoya, Japan; All other chemicals, unless otherwise stated, were obtained from sigma chemicals, St. Louis, MO, USA.

2.2. Cells and cell culture conditions

Human hepatocellular carcinoma cell lines, Bel7402 and HepG2, were obtained from National Center for Pharmaceutical Screening, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China. L02 cells were obtained from RIKEN Cell Bank, Ibaraki, Japan. Bel7402 cells were incubated in RPMI 1640 medium supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 in air at 37 °C. The HepG2 and L02 cells were incubated in DMEM medium supplemented with 10% FBS in a humidified atmosphere with 5% CO_2 in air at 37 °C. Then the cells in logarithmic growth phase were collected for the following experiments.

2.3. Cell proliferation assay for 5-FU, luteolin and their combination

The effects of luteolin, 5-FU, and their combinations on the growth of Bel7402, HepG2 and L02 cells were evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS)/phenazine methosulfate (PMS) assay. Cells in logarithmic growth phase were plated in 96-well plates at a density of 3×10^3 cells/well. After 24 h incubation, the cells were incubated with different concentrations of 5-FU (final concentration: 10, 5, 2.5, 1.25, 0.5, 0.25 μg/mL), or luteolin (final concentration: 100, 50, 25, 12.5, 6.25, 3.125 μM) for 21, 45 and 69 h. Then MTS and PMS (20:1 mixture) were immediately added to the culture medium at a ratio of 1:5. Three hours later, formazan production was analyzed at 490 nm in a plate reader (Molecular Devices, LLC). The inhibitory rates and IC_{50} values were then calculated.

For drug combinations, the cells were pretreated with luteolin for 2 h, and then 5-FU was added. The combined drug effects were determined using the isobole analysis [17]. Cells were treated with combined luteolin and 5-FU at fixed dose ratios of 10:1 (2.5 μM:0.25 μg/mL, 5 μM:0.5 μg/mL, 10 μM:1.0 μg/mL, 20 μM:2.0 μg/mL, 40 μM:4.0 μg/mL), 20:1 (2.5 μM:0.125 μg/mL, 5 μM:0.25 μg/mL, 10 μM:0.5 μg/mL, 20 μM:1.0 μg/mL, 40 μM:2.0 μg/mL), and 40:1 (5 μM:0.125 μg/mL, 10 μM:0.25 μg/mL, 20 μM:0.5 μg/mL, 40 μM:1.0 μg/mL, 80 μM:2.0 μg/mL) for 45 h, and then determined by MTS/PMS method. The drug concentrations of single or combined drugs that produced a 50% inhibitory effect were used as coordinates to construct isobolograms [20]. The diagonal straight line connecting the IC_{50} of luteolin and 5-FU represents the theoretical line of additivity for a continuum of different fixed dose ratios. When the combination is synergistic, the isobole is a concave curve. And an antagonistic combination yields a convex curve. The interaction index (I) defined synergistic (<1), additive ($=1$) and antagonist (>1) effects.

2.4. Cell viability and apoptosis

Bel7402, HepG2 and L02 cells in logarithmic growth phase were plated in 96-well plates at a density of 3×10^3 cells/well. After 24 h incubation, the cells were incubated with 5-FU (1.0 μg/mL), luteolin (20 μM), and their combinations for 46 h. After treatment, CellTiter-Blue Reagent (1:5) was added into each well. Then the plates were shaken for 10 s and incubated for 2 h. Then, fluorescence was detected at 560/590 nm. After CellTiter-Blue detection, the mixed Caspase-3/7 Glo reagent was added (1:1) into the same wells, and incubated for 1 h. Finally, the fluorescence was detected at 499/521 nm in a plate reader.

2.5. Cell-cycle analysis using flow cytometry

Cell-cycle perturbations were measured using flow cytometry after PI staining. Briefly, HepG2 and Bel7402 cells were cultured in 60 mm plates (3×10^5 cells/well) overnight and then in culture medium without FBS for an additional 24 h. Then cells were treated with luteolin (20 μM), 5-FU (1.0 μg/mL) and their combinations for 48 h. The cells were then harvested, washed twice with PBS, and fixed in 70% cold ethanol overnight. Before detection, cells were washed twice with cold PBS, and stained with 50 μg/mL of PI in the presence of 50 μg/mL of RNase A at 37 °C for 30 min. The cells were analyzed by flow cytometry in a FACScalibur and CellQuestPro software (BD Biosciences, Le Pont de Claix, France). PI fluorescence was collected with a 585 ± 44 nm filter.

2.6. Hoechst 33342 staining

Hoechst 33342 staining was used to observe the apoptotic morphology of the treated cells. Briefly, cells were seeded in 48-well plates at 2×10^4 cells/well. After overnight incubation, the cells were treated with luteolin (20 μM), 5-FU (1.0 μg/mL) and their combinations for 48 h. Then the cells were washed and stained by Hoechst 33342 (10 μg/mL) in culture medium containing 1% FBS at 37 °C for 60 min. Finally, after being washed with PBS, morphologic changes of the cells were observed under a fluorescence microscope and photographed.

2.7. Annexin V-FITC/PI analysis for apoptosis

Apoptosis was quantified using annexin V-FITC/PI staining kit according to the manufacturer's instruction. Briefly, cells were seeded in 60 mm culture dishes at a density of 3×10^5 cells/mL. After overnight incubation, the cells were treated with luteolin (20 μM), 5-FU (1.0 μg/mL), and their combinations for 48 h. Then cells were collected and analyzed with annexin V-FITC/PI staining by flow cytometry in a FACScalibur and CellQuestPro software.

2.8. Measurement of mitochondrial membrane potential ($\Delta\psi m$)

$\Delta\psi m$ was measured by MitoCapture™ Mitochondrial Apoptosis Detection Kit according to the manufacturer's protocols. Cells were seeded in 24-well plates at a density of 2×10^4 cells/mL. After treatment with 1.0 μg/mL 5-FU, 20 μM luteolin, and their combinations for 48 h, 500 μL diluted MitoCapture Reagent (1:1000 dilution, prepared just before use) was added to each well. Then cells were incubated at 37 °C in a 5% CO_2 incubator for 15 min. After that, the supernatant was discarded