SUPPLEMENTARY MATERIAL

Combinatorial anticancer effects of curcumin and Sorafenib towards thyroid cancer cells via PI3K/Akt and ERK pathways

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Abstract

The objective of this study was to examine the in vitro combinatorial anticancer effects of curcumin and Sorafenib towards thyroid cancer cells FTC133 using a MTT cytotoxicity assay, and to test whether the mechanism involves induction of apoptosis. The present results demonstrated that curcumin at 15 to 25 μ M dose-dependently suppressed the proliferation of FTC133. Combined treatment (curcumin (25 μ M) and Sorafenib (2 μ M)) resulted in a reduction in cell colony formation and significantly decreased the invasion and migration of FTC133 cells compared with that treated with individual drugs. Western blot showed that the levels of p-ERK and p-Akt proteins were significantly reduced ($P < 0.01$) in the medicine-treated FTC133 cells. The curcumin was found to dose-dependently inhibit the apoptosis of FTC133 cells possibly via PI3K/Akt and ERK pathways. There is a synergetic antitumour effect between curcumin and Sorafenib.

Keywords: curcumin, Sorafenib, Antitumour, PI3K/Akt and ERK pathways

1. Experimental

1.1. Material

Curcumin was purchased from Sigma. Purity is 99%.

Sorafenib was purchased from Beijing Baier Health care limited company (Beijing, China).

1.2. MTT assay

MTT assay was performed by the method described previously. Briefly, FTC133 in suspension were seeded at 1×10^4 cells per well in 96-well microtiter plate and these cells were grown in a humidified atmosphere of 5% CO₂ in air at 37 °C (Lab-Line CO₂ Incubator, Merlose Park, USA). Then the cells were exposed to varying concentrations of the curcumin (15, 25 µM) and curcumin (25 µM)+ Sorafenib (2 µM) for 48 h. After that, culture medium was carefully removed and exchanged for a fresh one. MTT solution (5 mg/ml PBS) was then added and the plate was located in optimal atmosphere at 37 °C. The metabolically active cells reduced MTT to blue formazan crystals. After 2 h, MTT-formazan crystals were dissolved overnight in 20% SDS and 50% DMF at pH 4.7 and absorbance was measured at 570 nm on a multifunctional plate reader (Victor 3, Perkin-Elmer, Finland) and compared with control, untreated cells.

The values of IC₅₀ (the concentrations of test compound required to reduce the survival of cells by 50%) were calculated from concentration–response curves and used as a measure of cellular sensitivity to a given treatment.

1.3. Annexin V-FITC/PI analysis for apoptosis

Apoptosis was quantified by annexin V-FITC/PI staining kit. Briefly, after the cells were treated with curcumin (15, 25 µM) and curcumin (25 µM)+ Sorafenib (2 µM) for 48 h, cells were collected and stained with annexin V-FITC and PI according to the manufacturer's instruction. Finally, the cells were analyzed using Flow cytometer (Becton Dickinson) and WinMDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

1.4. Transwell Assays for Cellular Migration, and Invasion

FTC133 cells were resuspended in serum-free culture medium and placed in the upper chamber of the transwell insert (1×10^6 cells/well) with varying concentrations of curcumin (15, 25 µM) and curcumin (25 µM)+ Sorafenib (2 µM). Before use, filters were pre-coated for 10 hours at 4°C with fibronectin (10 µg/ml; Sigma- Aldrich) and washed thrice. Cells were allowed to migrate in 5% CO₂ for 48 hours at 37°C, fixed with methanol for 10 minutes at room temperature and stained with 0.1% crystal violet. The underside of the filters was examined with a 40 × objective of a Nikon Eclipse T-200 (Tokyo, Japan) inverted phase-contrast microscope and number of migrating cells was determined for each well. For cell invasion assay, the procedure was the same with the modification that the upper chamber was coated with Matrigel (Becton-Dickinson) and cells were let to invade through it. Each experiment was done 3 times in triplicates and measurements represent the average

1.5. Colony forming efficiency assay

Colony forming efficiency assay was performed to evaluate the effectiveness of the tested agents against long-term cancer cell proliferation. FTC133 cells were cultured, suspended in RPMI1640 with 10% pasteurized FCS, and seeded in a 24-well flat-bottomed plate filled with 200 cells/ml and stabilized by incubation for 24 h at 37 °C. Then, curcumin (15, 25 µM) and curcumin (25 µM)+ Sorafenib (2 µM) were added and incubation at 37 °C. After the medium was replaced by the fresh medium,

incubation was continued for a total incubation period of 14 days. The medium was removed from the cells, which were successively washed with PBS and immobilized with MeOH. After removing MeOH, the cells were stained with Giemsa staining solution for 30 min, excess stain was washed with PBS solution and colonies (>50 cells) were counted using a light microscope. Plating efficiency of each treatment was calculated as the following formula:

$$\text{Colony form rate \%} = \frac{\text{number of viable colonies formed}}{\text{total number of cells plated}} \times 100\%$$

1.6. Western blotting

FTC133 cells were pretreated with curcumin (15, 25 μM) and curcumin (25 μM)+ Sorafenib (2 μM) for 48 h. The harvested cells were disrupted, and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories), as described in the product manual. Lysate protein (20 μg) was subjected to 10% SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane. After blotting, the membrane was incubated overnight with a specific primary antibody at 4 °C. Protein bands were visualised with a chemiluminescence detection kit (Amersham Pharmacia Biotech) after hybridization with a horseradish-peroxidase-conjugated secondary antibody.

Figure S1. Effect of curcumin and Sorafenib on FTC133 cells growth

** $P < 0.01$, compared with control (0 $\mu\text{g}/\mu\text{L}$); Data depict mean values $\pm$ SE from triplicates.

Figure S2. Effect of curcumin and Sorafenib on FTC133 cells apoptosis

** $P < 0.01$, compared with control (0 μM); Data depict mean values $\pm$ SE from triplicates.

Figure S3. Effect of curcumin and Sorafenib on colony forming efficiency (%) of FTC133 cells

** $P < 0.01$, compared with control (0 μM); Data depict mean values $\pm$ SE from triplicates.

Figure S4. Effect of curcumin and Sorafenib on FTC133 cells migration and invasion
 ** $P < 0.01$, compared with control (0 μM); Data depict mean values $\pm$ SE from triplicates.

Figure S5. Effect of curcumin and Sorafenib on ERK and p-ERK proteins expression in FTC133 cells

****** $P<0.01$, compared with control (0 μM); Data depict mean values $\pm$ SE from triplicates.

Figure S6. Effect of curcumin and Sorafenib on PI3K, Akt and p-Akt proteins expression in FTC133 cells

****** $P<0.01$, compared with control (0 μM); Data depict mean values $\pm$ SE from triplicates.