RESEARCH ARTICLE

Chloroquine and Valproic Acid Combined Treatment *in Vitro* has Enhanced Cytotoxicity in an Osteosarcoma Cell Line

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Abstract

Choroquine (CQ) and valproic acid (VPA) have been extensively studied for biological effects. Here, we focused on efficacy of combined CQ and VPA on osteosarcoma cell lines. Viability of osteosarcoma cell lines (U20S and HOS) was analyzed by MTT assay. Apoptotic assays and colony formation assays were also applied. ROS generation and Western Blotting were performed to determine the mechanism of CQ and VPA combination in the process of apoptosis. The viability of different osteosarcoma cell lines significantly decreased after CQ and VPA combination treatment compared with either drug used alone, and apoptosis was increased significantly. ROS generation was triggered leading to expression of apoptosis related genes being increased and of anti-apoptotic related genes being decreased. From our data shown here, CQ and VPA combination treatment in vitro enhanced cytotoxicy to osteosarcoma cells.

Keywords: Choroquine - valproic acid - proliferation - apoptosis - osteosarcoma cells

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Introduction

The common malignant bone tumor, especially in children and adolescents, is osteosarcoma (Li et al., 2013; Liu et al., 2013; Sampson et al., 2013). However, in present clinical therapeutic method, chemotherapy is still in low efficiency and causegreat pain to the patient (Chen et al., 2013; Sangsin et al., 2013). Therefore, new drugs or treatment for anti-osteosarcoma therapy are needed to be developed.

Chloroquine (7chloro4[4diethylamino1methyl butylamino] quinoline; CQ) is commonly used as anti-malaria and antiinflammation drug for rheumatoid arthritis, amoebic hepatitis and discoid lupus erythematosus (Wang et al., 2011; Harhaji-Trajkovic et al., 2012; Zinn et al., 2013). Recent report has shown that CQ has direct cytotoxicity and can enhance the cytotoxicity of radiotherapy. The cytotoxicity of CQ has been examined in breast, lung, colon, hepatic, pancreatic, ovarian cancers and glioma cell lines (Verschooten et al., 2012; Enzenmüller et al., 2013; Gao et al., 2013; Seitz et al., 2013). Clinical trial demonstrated that CQ could improve the midterm survival when given in addition to conventional chemotherapy and radiotherapy for glioblastoma multiforme (Verschooten et al., 2012; Enzenmüller et al., 2013). Nevertheless, the relevant mechanisms of the enhanced effect of CQ are still unclear.

A potent and specific inhibitor of HDAC, valproic acid (VPA), newly report have shown that it can suppress a broad spectrum of cancer cells (Shan et al., 2012; Sidana et al., 2012; Jasek et al., 2012; Kwiecińska et al., 2012; Li et al., 2013; Mackenzie et al., 2013) through the increase histone acetylation and apoptosis related gene expression activation, but the detailed mechanisms are still not clearly (Kwiecińska et al., 2011; Xie et al., 2012; McCormack et al., 2013; Nie et al., 2013).

If CQ can enhance the efficacy of VPA at lower concentrations, CQ and VPA might be cancer treatment because of their safety and lower cost. Therefore, the present study aims to evaluate the anti-osteosarcoma potential of CQ and VPA combination.

Materials and Methods

Drugs and reagents

Dimethyl sulfoxide (DMSO), cisplatin and propidium iodide (PI) were purchased from Sigma-Aldrich Chemical. Antibodies against Bax, Bcl-2, Bcl-XL, Bak, cytochrome c, β-actin, peroxidase-labeled anti-rabbit immunoglobulin G (IgG), and ECL Western blotting system were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). We dissolved CQ and VPA (Sigma-Aldrich, USA) in distilled water at a stock concentration of 1 M and filtered through a 0.2-mm filter.

The osteosarcoma cell lines (U20S, HOS, MG-63, LM8 and SaOS-2) were obtained from the American Type Culture Collection (ATCC, USA), and cultured in RPMI 1640 medium (Gibco, USA) supplemented with

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10% fetal calf serum. For drug treatment, cells were grown to about 90% confluency and detached by 0.5% ethylenediaminetetraacetic acid (EDTA)-trypsin.

Cell viability assay

Cell viability was determined using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. U20S and HOS cells were plated in 96-well plate. After 16 h attachment, the medium was replaced with fresh medium at various concentrations of drugs (CQ, VPA and/or its combination) with different incubation time. After treatment over, 20 μl of 5 mg/ml MTT was added to each well for an additional 4 h, then the medium was removed and 100 μl DMSO was added. The absorbance of samples was measured at 570 nm with a SpecTRA MAX microplate reader (Molecular Devices, USA).

Annexin V/PI assay

Annexin V/PI assay was used to detect apoptotic cells by staining cells with annexin V-FITC and PI. Cells were treated with CQ, VPA and combination respectively. After 48 h treatment over, 2×10^6 cells/ml were washed twice with PBS and resuspended in 300 μl binding buffer [10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, pH 7.4]. Three microliters of annexin V-FITC was added to the cells. Cells were incubated on ice in the dark for 1.5 h and washed with 4 ml HEPES buffer once. Ten microliters of PI (50 μg/ml) was added to these cells. Cells were incubated for another 20 min and then analyzed within 30 min by FACSCalibur (Becton Dickinson, USA). The same experiments were repeated for three times.

Caspase-3 activity assay

The activity of the caspase-3 was examined using a Diagnostic Reagent kit (Nanjing Jiancheng) following the manufacturer's instructions. Briefly, treated by drugs and untreated U20S and HOS cells were washed with PBS, cells were harvested in lysis buffer on an ice bath. Cell lysate were centrifuged at 12,000 × g for 10 min, and 200 μg of proteins was incubated with appropriate protease assay buffer and appropriate substrate, respectively, at 37 °C for 4 h. Optical density of the reaction mixture was detected by a spectrophotometer at the wavelength of 405 nm. Experiments were performed three times.

Colony formation assay

U20S, HOS and SaOS-2 cells were seeded into 35 mm dishes. After 6 h adherence, cells were treated with CQ, VPA and/or combination. Seven days later, colonies were fixed with methanol and stained with 1.25% Giemsa and 0.125% crystal violet for counting. Colony formation was expressed in relation to the control. Values are given as mean of three independent experiments.

Intracellular reactive oxygen species (ROS) assay

ROS generation was analyzed using DCFH-DA dye. Briefly, U20S and HOS cells (2×10^4) were seeded in one well of 96-well black bottom plate; adhered for 24 hours in a CO_2 incubator at 37 °C. The cells were then challenged with CQ and VPA for 24 h. Discard medium, incubated with DCFH-DA (10 μM, Ex/Em = 485 nm/528 nm) for 30 minutes at 37 °C in the dark. Aspirate reaction mixture and replaced by PBS (200 μL per well). Keep the plate on a shaker for 10 min at room temperature in the dark. Fluorescence intensity was measured using a Multiwell microplate reader (FLUOstar), and the values were expressed as a percentage of fuorescence intensity relative to the control wells.

Western blot analysis

After the indicated treatments over, cell lysates were prepared, separated and analyzed. In brief, after determination of the protein concentration using the BCA Protein Assay Kit (Biosynthesis Biotechnology Co., Ltd., China), equal amounts of protein (40 mg) from each sample were separated by electrophoresis through SDS-PAGE gels and transferred to PVDF membrane. Protein bands were visualized using enhanced chemiluminescence.

Statistical analysis

The data were expressed as means ± S.D. Statistical analysis was performed by using Student's t-test. The criterion or statistical significance was taken as ($p < 0.05$).

Results

CQ and VPA combination significantly suppress the proliferation of osteosarcoma cell lines

To assess whether CQ and VPA could affect viability of osteosarcoma cell lines, we initially treated two osteosarcoma cell lines (U20S and HOS) with CQ, VPA or the combination of both agents. Quantitative assessment of viable cells was performed at 12, 24, 48 and 72 hours using a standard MTT assay. In Figure 1, the growth of U20S and HOS cells was significantly inhibited by CQ (Figure 1A) and VPA (Figure 1B) in a dose and time

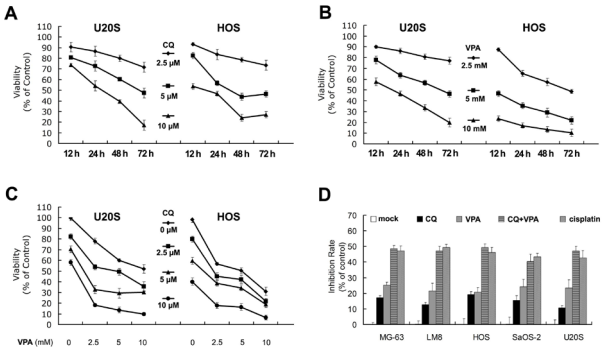

Figure 1. Cell Viability Assay of CQ and VPA Combination on Osteosarcoma Cells. (A) Viability of U20S and HOS cells treated by CQ at the concentration of 2.5, 5 and 10 μM for 12, 24, 48 and 72 hours. (B) Viability of U20S and HOS cells treated by VPA at the concentration of 2.5, 5 and 10 mM for 12, 24, 48 and 72 hours. (C) Viability of U20S and HOS cells treated by CQ (2.5, 5 and 10 μM) and VPA (2.5, 5 and 10 mM) combination at the concentration of for 6 hours. (D) Inhibition rate of CQ (5 μM), VPA (5 mM VPA) alone or its combination (5 μM CQ plus 5 mM VPA) on other osteosarcoma cells (MG-63, U20S, LM8, SaOS-2 and HOS) for 6 hours treatment ($P < 0.05$)

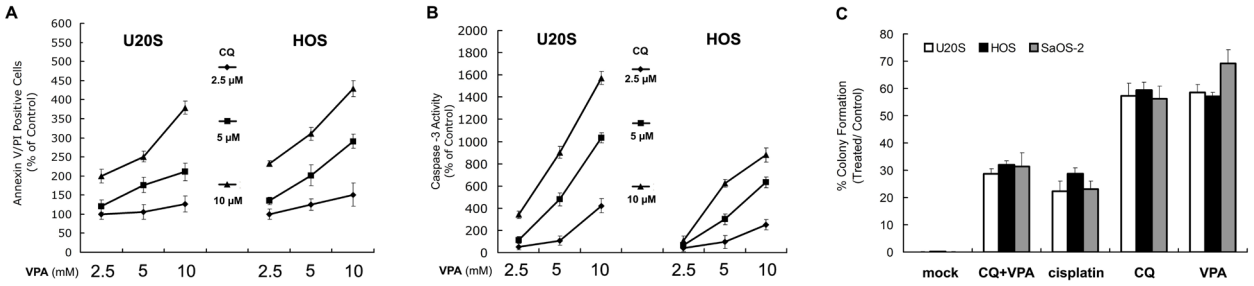

Figure 2. Apoptosis Assay and Colony Formation Assay on Osteosarcoma Cells Treated by CQ, VPA Alone or Its Combination. (A) Apoptotic rate of U20S and HOS treated by CQ (2.5, 5 and 10 μM), VPA (2.5, 5 and 10 mM) alone or its combination for 24 hours. (B) Caspase-3 activity determination after CQ (2.5, 5 and 10 μM), VPA (2.5, 5 and 10 mM) alone or its combination treatment for 24 hours. (C) Colony formation of U20S, HOS and SaOS-2 cells after CQ (5 μM), VPA (5 mM VPA) alone or its combination (5 μM CQ plus 5 mM VPA)

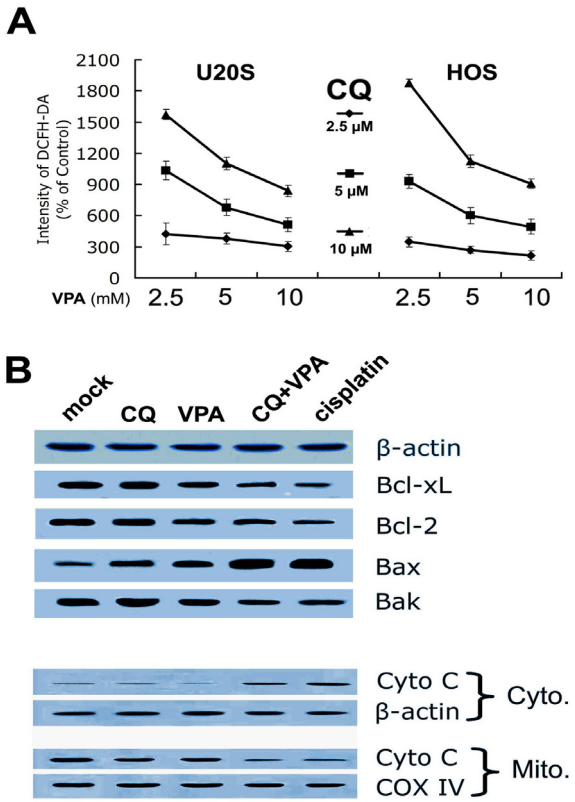

Figure 3. ROS Generation and Western Blotting Assay after Drug Treatment on U20S or HOS Cells. (A) ROS generation assay after CQ (2.5, 5 and 10 μM), VPA (2.5, 5 and 10 mM) alone or its combination treatment for 24 hours. (B) After CQ (5 μM), VPA (5 mM VPA) alone or its combination (5 μM CQ plus 5 mM VPA) treatment for 24 hours on U20S cells, apoptosis related genes expression level detected by Western blotting

dependent manner. In all two cell lines, the combination of CQ and VPA significantly decreased cell viability relative to either agent alone ($P < 0.05$, Figure 1C). Moreover, other osteosarcoma cell line MG-63, LM8 and SaOS-2 cells growth also significantly suppressed by CQ and VPA in combination (Figure 1D) ($P < 0.05$).

CQ and VPA significantly induce osteosarcoma cell lines apoptosis

We next sought to characterize whether the combination effects observed in osteosarcoma cells treated with CQ and VPA were associated with enhanced apoptosis, as hypothesized. Apoptosis assay by flow cytometry were used after drug treatment, the number of cells in apoptotic (Figure 2A) were also significantly increased when compared to drugs used alone ($P < 0.05$). Caspase-3 activation is classic marker of apoptotic induction through both the extrinsic and intrinsic apoptotic pathways. The combination of CQ and VPA resulted in higher levels of caspase-3 activation relative to either treatment alone in U20S and HOS lines (Figure 2B). Moreover, the CQ and VPA combination decreased the number of the colonies (Figure 2C) from U20S and HOS cell lines.

Classic apoptotic-related signaling triggered by CQ and VPA combination

ROS generation is also a hallmark of apoptotic induction. ROS was measured based on the increasing of fluorescence intensity quantification by monitor the enzymatic cleavage of DCFH-DA. In combination exposure, ROS generation significantly increased in relative ROS were observed at 24 h (Figure 3A). From the Western blotting assay, the combination drug treatment resulted in Bax increase, whereas Bcl-2, Bcl-xL, Bak decrease; moreover, cytochrome c (Cyt. c) significantly release from mitochondria into the cytosol (Figure 3B). That means that CQ and VPA combination significantly induce osteosarcoma cells apoptosis compared with drug used alone.

Discussion

Osteosarcoma is a bone forming cancer. It is the most frequent type of bone tumor between the ages of 15 to 25.The most common sites are the bones around the knee (Chen et al., 2013; Li et al., 2013; Liu et al., 2013; Sampson et al., 2013; Sangsin et al., 2013). Although extensive efforts have been made in anti-osteosarcoma therapy in recent years, there are still no effective low-toxicity drugs for treating osteosarcoma. Therefore, potent anti-metastatic osteosarcoma drugs are highly desired.

CQ and VPA has been used in cancer cell treating research, however, both of them have anti-tumor effect in high concentration with low efficiency respectively (Jasek et al., 2012; Zinn et al., 2013). In order to promote the efficacy of VPA, we combined CQ, a well-known anti-malarial agent, which inhibits lysosomal acidification and blocks the terminal stage of autophagy.

In summary, from our data shown here, CQ and VPA combined treatment in vitro significantly enhanced cytotoxic of osteosarcoma cells, including the proliferation suppression, apoptosiss induction and colony formation ability decreasing. During the cytotoxic effect, ROS generation was triggered leading to the apoptosis related genes expression increasing and anti-apoptotic related genes decreasing. The combination of CQ and VPA show significant inhibition of the growth of different osteosarcom cell lines such as U20S, HOS, MG-63, LM8 and SaOS-2. All these findings suggest that the CQ and VPA combination may have the therapeutic potential for anti-osteosarcoma therapy in clinical.

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The author(s) declare that they have no competing interests.

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