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Sorafenib potentiates ABT-737-induced apoptosis in human oral cancer cells

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ABSTRACT

Objective: The mimetic BH3 ABT-737, a potent inhibitor of anti-apoptotic Bcl-2 family proteins, has potential as anti-cancer drug in many cancers. Recently, patients treated with ABT-737 have developed drug tolerance during cancer therapy. Therefore, we examined whether ABT-737 is effective in killing MC-3 and HSC-3 human oral cancer cells either alone or in combination with the oncogenic kinase inhibitor, sorafenib.
Design: The potentiating activities of sorafenib in ABT-737-induced apoptosis were determined using trypan blue exclusion assay, DAPI staining, cell viability assay and Western blot analysis.
Results: Combined use of ABT-737 and sorafenib synergistically suppressed cell viability and induced apoptosis compared with either compound individually. The combination of ABT-737 and sorafenib altered only Bax and Bak proteins and their activations, resulting in mitochondrial translocation of Bax from the cytosol. Additionally, combination treatment-mediated apoptosis may be correlated with ERK and STAT3 pathways.
Conclusions: These results suggest that sorafenib may effectively overcome ABT-737 resistance to apoptotic cell death, which can be a new potential chemotherapeutic strategy against human oral cancer.
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1. Introduction

Proteins in Bcl-2 family member, critical regulators of apoptotic cell death, govern whether a cell will live or die through the interplay between anti-apoptotic and pro-apoptotic proteins (Oltersdorf et al., 2005). Anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL are over-expressed in various solid tumors and contributed to tumor development (Kirkin, Joos, & Zornig, 2004). ABT-737 is a small molecule that shows high affinity for the hydrophobic pocket groove of anti-apoptotic proteins (Ni Chonghaile & Letai, 2008). Since it has been discovered in 2005 as an inhibitor of Bcl-2 family proteins (Oltersdorf et al., 2005), numerous studies have demonstrated that ABT-737 displayed the anticancer potential as a single treatment for many types of cancers (Hann et al., 2008; Konopleva et al., 2006; Tagscherer et al., 2008; Tahir et al., 2007; Trudel et al., 2007; van Delft et al., 2006). Recently, our group also reported that ABT-737 induced apoptosis via the transcriptional or post-translational modifications of Bim in human oral cancer cell lines and tumor xenograft (Shin et al., 2015). However, several other studies also demonstrated that a long-term treatment of ABT-737 made tumor cell lines resistant to this drug (Hikita et al., 2010; Konopleva et al., 2006; Yecies, Carlson, Deng, & Letai, 2010). Given that single treatment of ABT-737 causes drug resistance, it is important to identify a new therapeutic strategy to kill oral cancer.

Sorafenib (Nexavar, BAY43-9006), a multi-tyrosine kinase inhibitor, has shown to induce apoptosis in a wide variety of tumors (Liu et al., 2006; Rahmani, Davis, Bauer, Dent, & Grant, 2005; Ullen et al., 2010; Yu et al., 2005). We recently reported that sorafenib diminished the expression level of Mcl-1 protein by dephosphorylating STAT3 leading to apoptosis in human oral

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cancer cells suggesting that sorafenib can be an effective drug candidate against oral cancer (Yu et al., 2015). Recently, Lian et al. (2012) showed that sorafenib potentiated Gossypol (a natural BH3 mimetic)-induced growth suppression in prostate cancer cells. It suggests that sorafenib can be used in combination with additional chemotherapeutic drugs like the inhibitors of Bcl-2 family members.

In the present study, we hypothesized that sorafenib may enhance ABT-737-exerted antitumor activity in human oral cancer cells. Therefore, we verified the efficacy and molecular target of combination treatment of ABT-737 and sorafenib in human oral cancer cells *in vitro*.

2. Materials and methods

2.1. Cell culture and chemical treatment

MC-3 (human mucoepidermoid carcinoma) cells were provided by Prof. Wu Junzheng (Forth Military Medical University, Xi'an, China) and HSC-3 (human oral squamous carcinoma) cells were provided by Prof. Masanobu Shindoh (Hokkaido University, Hokkaido, Japan). Cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37 °C in a 5% CO_2 incubator. ABT-737 (Selleck Chemicals, Houston, TX, USA) and sorafenib (LC Laboratories, Woburn, MA, USA) were dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20 °C. The final concentration of DMSO did not exceed 0.1%.

2.2. Trypan blue exclusion assay

The growth inhibitory effect of combinational treatment of ABT-737 with sorafenib was determined using trypan blue solution (Gibco, Paisley, UK). Cells were stained with trypan blue (0.4%), and viable cells were then counted using a hemocytometer.

2.3. 4′-6-diamidino-2-phenylindole (DAPI) staining

To detect the nuclear morphological changes of apoptotic cells, cells were stained with DAPI solution (Sigma-Aldrich, Louis, MO, USA). Briefly, cells were fixed in 100% methanol at room temperature (RT) for 10 min, deposited on slides, and stained with DAPI solution (2 μg/ml). The morphological changes of apoptotic cells were observed under a fluorescence microscope (Leica microsystems, Wetzlar, Germany).

2.4. Cell viability assay

The cytotoxicity of combinational treatment of ABT-737 with sorafenib was determined using the Live/Dead Viability/Cytotoxicity assay (Life Technologies, Grand Island, NY, USA). Briefly, cells were stained with 2 μM Calcein-AM and 4 μM Ethidium homodimer-1 and then incubated for 30 min at RT. Cells were analyzed under a fluorescence microscope with the appropriate excitation and emission filters.

2.5. Western blot analysis

Whole-cell lysates were prepared with lysis buffer and the protein concentration of each sample was measured using a *DC* Protein Assay Kit (Bio-Rad Laboratories, Madison, WI, USA). After normalization, equal amount of proteins were separated by SDS-PAGE and transferred to Immun-Blot™ PVDF membranes. The membranes were blocked with 5% skim milk in TBST at RT for 2 h and incubated with primary antibodies and the corresponding HRP-conjugated secondary antibodies. Antibodies against cleaved PARP, cleaved caspse-3, Bax, Bak, Bcl-2, Bcl-XL, Mcl-1, p-ERK (Thr202/Tyr204), ERK, p-STAT3, STAT3, p-AKT and AKT were purchased from Cell Signaling Technology (Charlottesville, VA, USA). The Bax (6A7) antibody was purchased from BD Pharmingen™ (San Jose, CA, USA) and the Cox4 antibody was purchased from Abcam (Cambridge, UK). Actin and α-tubulin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The immunoreactive bands were visualized using an ImageQuant™ LAS 500 system (GE Healthcare Life Sciences, Piscataway, NJ, USA).

2.6. Statistical analysis

A Student's *t*-test was used to determine the significance of differences between the control and treatment groups; values of $p < 0.05$ were considered significant.

3. Results

3.1. Sorafenib synergistically kills oral cancer cells when combined with ABT-737

To examine the effects of either ABT-737 alone or in combination with sorafenib in human oral cancer cells, we first carried out a trypan blue exclusion assay. We found that either ABT-737 or sorafenib slightly suppressed the growth of MC-3 cells, but the combination treatment displayed strong synergistic growth-inhibitory activity against MC-3 cells (Fig. 1A). We then performed western blot analysis using antibodies directed against PARP and caspase 3 to determine whether the growth inhibitory

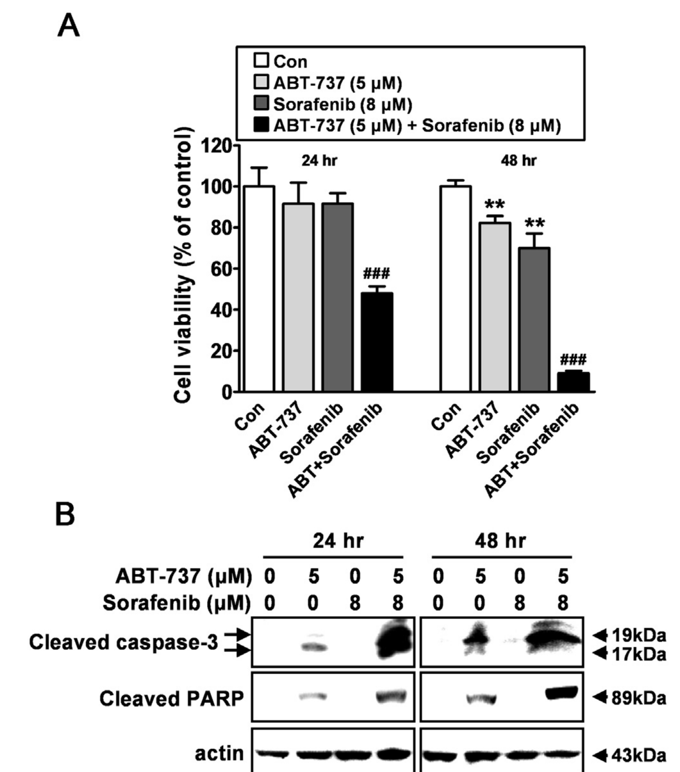

Fig. 1. Combined use of ABT-737 and sorafenib inhibits cell viability and induces apoptosis in human oral cancer cells.
MC-3 cells were treated with indicated compounds for 24 and 48 h. (A) Cell viability was determined using a trypan blue exclusion assay. The graphs were shown the mean ± S.D. of triplicate experiments. Significance ($p < 0.01$) compared with the DMSO-treated group as indicated (**) and significance ($p < 0.001$) compared with the ABT-737 or sorafenib-treated group as indicated (###). (B) Whole-cell lysates were subjected to Western blot analysis with the indicated antibodies and normalized to actin.

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