Bufalin Enhances the Cytotoxicity of Human Multiple Myeloma Cells H929 to AKT Inhibitor MK2206: The Role of Protein AKT Phosphorylation

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Abstract This study was purposed to investigate bufalin combined with AKT inhibitor MK2206 on growth inhibition and apoptosis of multiple myeloma cell line H929. CCK-8 assay and Annexin/PI staining were used to access the effects of bufalin and MK2206 in single or in combination, on inhibition of proliferation and induction of apoptosis in H929 cells. The apoptotic cells markedly increased after treated with nM bufalin and μ M MK2206, including caspase3 and PARP1 proteins activated. The difference was statistically significant ($P < 0.05$) when compared with these drugs in single use. The apoptosis associated proteins and AKT/p-AKT proteins were determined by Western blots. We confirmed that AKT performed contradictory results in H929 with the two agents, and concluded p-AKT was vital in the synergy. The underlying mechanisms warrant further investigation.

Keywords Bufalin · AKT · p-AKT · Multiple myeloma

Introduction

Multiple myeloma (MM) as the second most common hematologic cancer in the world, has greatly improved the rates and depth of response with the introduction of several classes of new effective drugs over the past decade [1]. AKT contributes to multiple oncogenic functions in

multiple myeloma and AKT activity is important for MM cell expansion [2]. As a central node in the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway with high expression in advanced and resistant multiple myeloma, promising pre-clinical data has support for pursuing further development of AKT inhibitors in multiple myeloma. Lead drugs are now entering the clinic. Even experts indicated AKT inhibitors may be set to improve patient outcomes when used in combination with synergistic drug partners [3].

AKT activation mediates multiple biological activities including increased survival, proliferation, and growth of tumor cells, and the effect of AKT on survival of cancer cells is related to its antiapoptotic properties [4, 5]. MK2206 is an orally active allosteric AKT inhibitor that is a highly selective inhibitor of AKT1/2/3 and inhibits autophosphorylation of both AKT T308 and S473 [6]. MK2206 has been confirmed that its function as cytotoxicity in MM cell lines and patient cells tested is highly dependent on basal p-AKT levels. MK2206 inhibited p-AKT and its down-stream targets and up-regulated p-Erk in MM cells. The induction of anti-MM is due to cell apoptosis, which at least partially is mediated by caspases [7]. Bufalin is a cardiotonic steroid isolated from the skin and parotid venom glands of toads of the *Bufo* species (*Bufo bufo gargarizans*), which is commonly used in the practice of Chinese medicine. The antitumor activity of bufalin has been demonstrated in various cancer cells, including MM [8], leukemia [9–11], and cancers of the prostate [12], ovaries, endometrium [13], gastrointestinal system [14], pancreas, lung [15], and liver cells [16]. These antitumor effects have been mainly attributed to the induction of apoptosis and cell cycle arrest. Accumulating evidence on bufalin reveals that it stimulates reactive oxygen species and inhibits of NF- κ B, STAT3, and AKT signaling

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pathways, all of which are contributed to its antitumor effects. The Na^+/K^+ -ATPase has been shown to be a direct target of bufalin [17, 18].

Based on these evidences, we have examined the anti-MM effects of bufalin and MK2206 on H929 cell line proliferation and viability, respectively. Either bufalin or MK2206 was related to apoptosis, used in combination, they increased apoptosis rate. According to the p-AKT level tested, we proposed bufalin could enhance the cytotoxicity of human multiple myeloma cells H929 to AKT inhibitor MK2206.

Materials and Methods

Cell Culture

The human multiple myeloma cell lines NCI-H929 was purchased from American Type Culture Collection (ATCC). The cell culture was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; hyClone, Logan, UT), penicillin (100 IU/mL) and streptomycin (100 mg/mL) in a humidified incubator at 37 °C and 5% CO_2 /95% air. The cells were passaged twice weekly to maintain an exponential growth phase.

Small Molecular Compounds

Bufalin (Tauto Biotech Co., Ltd., Shanghai China) and MK2206 (Merck & Co. Inc) described in Fig. 1, were both dissolved in dimethyl sulfoxide (DMSO) to make stable stock solution and stored at −20 °C.

Cell Proliferation and Cell Apoptosis Assay

H929 cells ($0.1\text{--}3 \times 10^5$ per 200 μL) were seeded into 96-well plates and incubated with various drug concentrations in triplicates for 48 h. Cell counting kit-8 (CCK8) (Dojindo, Kumamoto, Japan) was used to measure cell proliferation as described previously [19]. Each experiment

was conducted in triplicate and repeated three times. Cell apoptosis was measured with the Annexin-V Apoptosis detection kit (BD Pharmingen) following the manufacturer's instructions. Annexin-V-positive and propidium iodide-negative cells were considered to be in the early apoptotic phase and those having positive staining both for Annexin-V and propidium iodide were considered to be in stages of late apoptosis or necrosis. All data were collected, stored, and analyzed by LYSIS II software (BD Biosciences).

Western Blot

H929 cells were harvested, washed with PBS and lysed with lysis buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol). Cell lysates were centrifuged at 20,000g for 10 min at 4 °C, and proteins in the supernatants were quantified. Protein extracts were equally loaded to 6–15% SDS-polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, United Kingdom). After blocking with 5% nonfat milk in PBS for 2 h at room temperature, the membranes were incubated with antibodies against p-AKT (Ser473), cleaved caspase3, (Cell Signaling, Beverly, MA) and PARP1 (Santa Cruz Biotechnology, CA) overnight at 4 °C, followed by HRP-linked secondary antibody for 1 h at room temperature. The signals were detected by chemiluminescence phototope-HRP kit (Millipore) according to manufacturer's instructions, and α -tubulin (Bioworld Technology, Inc) was probed as an internal control.

Statistical Analysis

All the experiments were carried out in triplicate. The results were calculated by using the Student *t* test. Data were presented as mean $\pm$ SD. Statistical significance was defined as $P < 0.01$.

Results

Bufalin and MK2206 Alone Can Inhibit H929 Cells Proliferation

To assess the effect of drugs on cell viability, H929 cells were treated with various concentrations of bufalin or MK2206 for 48 h, and CCK8 assay was performed to evaluate cell proliferation. According to the Fig. 2a, the cytotoxicity of drugs on the cell viability increased with the raised concentrations. Bufalin inhibited H929 cell growth with IC_{50} about 10–20 nM at 48 h, while MK2206 with IC_{50} about 5–10 μM . Comparing with agent independent,

Fig. 1 Chemical structures of bufalin and MK2206

Fig. 2 CCK-8 assay of H929 cells treated with drugs. **a** H929 cells were cultured with concentrations of bufalin or MK2206 for 48 h. **b** The growth of H929 cells were inhibited by 20 nM bufalin or 10 μ M MK2206 alone, or in combination for 48 h. All values represent means $\pm$ SD of three independent experiments, each performed in triplicate (** $P < 0.01$, compared to the agent alone)

the viability of cells significantly decreased in combined action (Fig. 2b).

Bufalin Enhances Cytotoxicity of MK2206 on H929 Cells

Establishing the combination effect of the two drugs, we next examined the apoptotic effects in H929 cells. Cells were treated with 20 nM bufalin, or 10 μ M MK2206, or both two drugs with the indicated concentrations for 48 h, and the percentage of apoptotic cells were further evaluated by AnnexinV/PI double staining. As shown in Fig. 3, bufalin and MK2206 treatment induced 17.86 ± 0.71 and $20.27 \pm 0.47\%$ Annexin-V positive cells in H929, respectively. And the obviously increased apoptosis rate

($66.48 \pm 0.45\%$; $P < 0.01$) confirmed synergistic interaction of MK2206 with bufalin.

The Combination of MK2206 and Bufalin Induces H929 Cells Apoptosis by Caspase Activation

To confirm the synergistic interaction of MK-2206 with bufalin, we evaluated the induction of apoptotic cell death in H929 cells (Fig. 4). MK2206 and bufalin alone at concentrations up to 10 μ M and 20 nM, respectively, did not induce a significant increase in activated caspase3. Particularly, MK2206 alone did not activate caspase3 in H929 cells when cultured for 48 h, suggesting MK2206 barely induced cell apoptosis in that condition. The combination of both agents induced apparent cleaved caspase3 and PARP1. Bufalin treatment alone could stimulate p-AKT

Fig. 3 Effect of bufalin and MK2206 on apoptosis in H929 cells. Values indicating Annexin V+/PI– and Annexin V+/PI+ cells detected by flow cytometry were expressed as mean $\pm$ SD of three independent experiments

Fig. 4 Western blot analysis of caspase3 and PARP1 in H929 cells treated with 20 nM bufalin and/or 10 μ M MK2206 for 48 h

expression, whereas MK2206 decreased the level of p-AKT, suggesting that bufalin has opposite result in H929 via AKT inhibition. These results indicate that the combination of these agents enhances cell death and that this enhancement leads, at least in part, to the synergistic induction of cell apoptosis.

Discussion

Our results revealed MK2206 and bufalin both had their own cytotoxicity on MM cell line H929, and they remarkably intensified H929 cells apoptosis in combination by activating apoptotic proteins caspase3 and PARP1. It was observed that the level of p-AKT expression in H929 increased following culture with bufalin.

The PI3K/AKT/mTOR pathway has long been shown to be important in MM disease progression and resistance to therapy, and AKT is a promising target for future molecular-based therapy. Here, we processed the study in H929 cell lines because the baseline levels of activated AKT were high. Previous investigation indicated that MM cell lines sensitive to MK2206 expressed high levels of p-AKT whereas the cell lines less sensitive to MK2206 expressed low levels of p-AKT. Furthermore, MK2206 was found to be extremely sensitive in killing MM cells with *t*(4;14) translocations (H929) irrespective of their p53 status (del17p) since H929 expresses wt p53 [7]. Accumulating

evidences on bufalin revealed that it stimulates reactive oxygen species and inhibits NF- κ B, STAT3, and AKT signaling pathways, all of which are contributed to its antitumor effects [20]. The Na⁺-K⁺-ATPase has been shown to be a direct target of bufalin, and our previous research indicated PARP1 was another target of bufalin [8]. In this study, we found bufalin could activate AKT and assist MM cells apoptosis by MK2206. Previous studies have reported that AKT could respond to DNA double-strand breaks (DSB) and actively stimulate the repair of DNA-DSB [21]. And small molecular compounds which can induce DNA damage were related to PI3K/AKT/mTOR signaling in multiple myeloma cells [22]. Thus, we consider the main reason of synergistic action between MK2206 and bufalin in H929 is blocking DNA damage repair. P-AKT expression as the feedback of DNA damage repair is stopped by MK2206, which results in cell injury. Then, bufalin not only intensifies DNA damage, but inhibits DNA damage repair [8]. Interestingly, bufalin activated AKT here in MM cell lines H929 whereas inhibited AKT-related signaling in most tumor cells as reported [20]. For the purpose to utilize agents in clinic, more researches in MM cell lines and patient cells (particular the cells with high levels of AKT expression) need to be carried out.

MK2206 is known as a new chemosensitizer, and bufalin as anti-tumor drug has not been familiarized even in China. In addition, bufalin as a Chinese traditional medicine, further work needs to be done in exploiting its anti-MM mechanism. Here, we just exhibited a special combination pattern and obvious anti-MM effect in single MM cell line H929. Taken together, our data suggests that AKT inhibitor MK2206 with bufalin provide an alternative approach for the treatment of MM. We realize that further studies are definitely required to understand the mechanism of this combination.

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Compliance with Ethical Standards

Conflict of interest All authors declare there is no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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