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Beta-Hydroxyisovalerylshikonin Inhibits the Growth of U266 Multiple Myeloma Cells by Triggering the Mitochondrial Pathway

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Abstract

Background: Beta-hydroxyisovalerylshikonin (beta-HIVS) is a compound isolated from the traditional oriental medicinal herb lithospermum radix. This drug exerts a role as an ATP non-competitive inhibitor of Protein-Tyrosine Kinases (PTKs) and shows great potential for induction of apoptosis against human cancer cells. We investigated the effect of beta-HIVS on multiple myeloma U266 cells and clarified details of the primary mechanism in its apoptosis-inducing activity.

Objective: The aim of this work was to trace the apoptosis-inducing activity of beta-HIVS on U266 cells as well as the underlying mechanisms.

Methods: Cell Counting Kit-8 (CCK-8) test and colony-forming assay were performed in estimating the effects of beta-HIVS on U266 cell viability and colony formation. Apoptosis analysis was carried out on the basis of DAPI fluorescence staining and DNA fragmentation assays. Real-time PCR was employed to evaluate changes of *Bcl-2* and *Bax* mRNA expression, while indirect immunofluorescence assay and western blotting were utilized in validating the expression of Bcl-2, Bax, caspase-3, caspase-9, PARP and cytochrome c.

Results: CCK-8 test and colony-forming assay showed that beta-HIVS treatment resulted in significantly reduced cell proliferation ($P<0.05$ or 0.01) and colony formation ($P<0.01$). Real-time PCR results indicated that the expression level of Bcl-2 mRNA was reduced at 72h following beta-HIVS co-cultivation ($P<0.01$), although *Bax* mRNA altered with no significance. Immunofluorescence assay displayed that caspase-3 was activated in beta-HIVS treated group, accompanied by an increased expression of cytochrome c. Western blotting also exhibited that the expression of Bcl-2 protein in beta-HIVS treated group decreased and cytochrome c increased at 72h after co-cultivation. Moreover, caspase-3 and -9, as well as PARP were activated, all with $P<0.01$ when compared with the two control groups.

Conclusion: Beta-HIVS revealed remarkable apoptosis-inducing activity in U266 cells, possibly by inhibiting proliferation and promoting apoptosis via the mitochondrial pathway.

Keywords: Beta-hydroxyisovalerylshikonin; Beta-HIVS; Multiple myeloma; U266 cell; Mitochondria pathway

Abbreviations: Beta-HIVS: Beta-HydroxyIsoValerylShikonin; MM:Multiple Myeloma; FBS: Fetal Bovine Serum; CCK-8: Cell Counting Kit-8; M: Mole/L

Introduction

Multiple Myeloma (MM) is a plasma cell malignancy accounts for more than 10% of hematological malignancies and is characterized by the proliferation of malignant clonal plasma cells that produce a single immunoglobulin isotype named M-protein [1,2]. At present, treatments using alkylating agents, corticosteroids, proteasome inhibitors and immunomodulatory drugs have improved the overall outcomes of MM during the past decade, and MM patients are now undergoing considerable survival rate with respect to those obtained with historical treatments. Nevertheless, MM remains in most cases an incurable disease, and novel drugs as well as therapeutic strategies are still required for continued disease control. In this regard, several new agents for MM therapy are currently receiving assessment, some of which seemed with promising on the basis of reported initial results [3-5].

Currently, a series of novel shikonin derivative analogues bearing oxygen-containing substituents were designed, synthesized or isolated [6-8], among which, beta-hydroxyisovalerylshikonin (beta-HIVS) has been highlighted for the strongest apoptosis-inducing activity among various derivatives of shikonin. This drug is an ATP non-competitive inhibitor of protein-tyrosine kinases and is capable of inducing apoptosis in various human tumor cell lines which has been documented by several publications [9-13]. It is also reported that beta-HIVS shows great promise as a potent apoptosis-inducing agent in the treatment of hematological malignancies included leukemia, although this still retains in research and development stages [14-16]. At present, the apoptosis-inducing activity of beta-HIVS on MM as well as the way in which beta-HIVS promotes cell death remains to be clarified. In consequence, the current study investigated the effect of beta-HIVS on U266 cells and traced the underlying mechanism.

Materials and Methods

Materials

The U266 cell line was purchased from the Chinese Academy of

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Sciences (Shanghai, China). The main reagents are listed as follows: beta-HIVS (Dibo Chem, Shanghai, China); RPMI-1640, fetal bovine serum (FBS) (Gibco-BRL, Carlsbad, CA, USA); DMSO, methylcellulose (Sigma-Aldrich Corporation, St. Louis, MA, USA); Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan); SYBR-Green PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany); RIPA protein lysis buffer, paraformaldehyde, DAPI dye, DNA Fragmentation kit, Cy3-conjugated anti-mouse IgG, FITC-conjugated anti-rabbit IgG, mouse anti-human β-actin, anti-cytochrome c, rabbit anticleaved PARP and anti-cleaved caspase-3 (Asp175) (Beyotime Institute of Biotechnology, Shanghai, China); rabbit antihuman Bcl-2 and anti-Bax (Abcam, Cambridge, MA,USA); rabbit anti-caspase-9 and anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA);

Methods

Cell culture

Human U266 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, and cultured at 37˚C in 5% CO_2.

Cell viability assays

Beta -HIVS was dissolved in DMSO with a stork concentration of 8000 μg/ml and the IC_{50} (1.2 μg/ml) for U266 cells was obtained by our previous study. Three groups as beta-HIVS treated group, DMSO control group and no treatment group were established. Cells were seeded in 96-well plates at a density of 5×10^3/ml and co-cultivated with beta-HIVS at a final concentration of 1.2 μg/ml. CCK-8 test was employed in the evaluation of the growth inhibitory effects of beta-HIVS on U266 cells following the manufacturer's protocol. The optical density (OD value proportional to the cell numbers) was measured using a microculture plate reader (BioTek Instruments, Winooski, VT, USA) at both 450 and 630 nm.

Colony-forming assay

Concisely, cells were suspended in a concentration of 300 cells per ml RPMI-1640 and seeded in 24-well plates. Methylcellulose dissolved in RPMI-1640 containing 30% FBS was supplied to each well at a concentration of 0.8 g/L. The colonies containing more than 50 cells were counted following incubation at 37˚C in 5% CO_2 for 14 days.

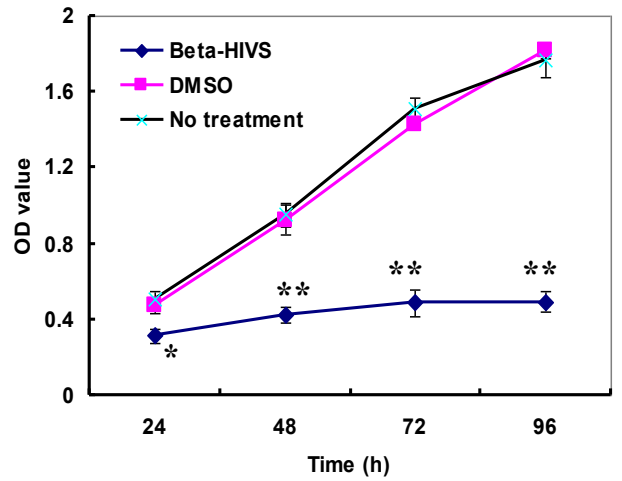

Figure 1a: Effects of beta-HIVS on the cell viability and colony formation of multiple myeloma U266 cells. (A) CCK-8 assay. The OD value proportional to the cell number was detected and plotted on the growth curve.

Apoptosis analysis

Cell apoptosis was evaluated via DAPI fluorescence staining and DNA fragmentation assays. For DAPI staining, cells were collected and washed with PBS and stained by DAPI (2 mg/ml) for 3-5 min. Morphological changes of the stained cells were examined using the fluorescence microscope (Olympus, Japan). (Non-viable apoptotic ratio) % = (cell numbers in phase IIb/200 cells) x100%. DNA fragmentation analysis was performed according to the instructions of a commercial DNA Fragmentation Kit, followed by agarose gel electrophoresis: each sample containing 2.5 μg of extracted DNA in 1.2% agarose gel and electrophoresis with a constant voltage 20 V for 4h.

Real-time PCR analysis

Real-time PCR test was carried out by utilizing the SYBR Green PCR Master Mix under the recommended conditions. The primer sequences for *Bcl-2*, *Bax* and *GAPDH* were previously described [17]. The comparative Ct method was used to calculate the relative expression level of *Bcl-2* or *Bax* as compared with *GAPDH*.

Immunofluorescence staining

The cell monolayer was fixed with 4% paraformaldehyde, and were incubated at 4˚C overnight with rabbit anti-cleaved caspase-3 (Asp175) (1:300) and mouse monoclonal anti-cytochrome c (1:400) antibodies. FITC-conjugated anti-rabbit and Cy3-conjugated anti-mouse IgG were all diluted at 1:1000. DAPI was used to dye the cell nuclei. The stained cells were washed with PBS and observed with a fluorescence microscope (Olympus, Japan) at x 400 magnification.

Western blotting analysis

Cell lysates were prepared with the aid of RIPA protein lysis buffer and the protein extracts were quantified and then subjected to electrophoresis on a 10-12% SDS-PAGE gel. The proteins were transferred onto the PolyVinylidene Difluoride (PVDF) membranes and blocked in Tris-buffered saline (TBS) containing 5% non-fat milk powder. The primary antibodies and the dilutions used were: rabbit anti-Bcl-2 (1:1000), anti-caspase-9 (1:500), anti-caspase-3 (1:500) and anti-cleaved PARP (1:300). Mouse monoclonal anti-cytochrome c antibody was diluted at 1:500 and mouse anti-β-actin antibody was 1:1000.

Statistical analysis

All data are expressed as the means ± Standard Deviation (SD). One-way ANOVA, Student's t-test or the non-parametric test were performed for comparison of differences between groups using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Beta -HIVS inhibits U266 cell viability and colony formation

As shown in (Figure 1A), the effects of beta -HIVS on the viability of U266 cells were assessed by CCK-8 assay. The results suggested that beta-HIVS treatment led to a detection of 0.312±0.035, 0.433±0.052, 0.491±0.047, and 0.489±0.076 of the OD values (proportional to the cell numbers) at 24, 48, 72 and 96 h following co- cultivation, significantly lower than those of DMSO or no treatment cells with $P<0.05$ or 0.01. For the colony formation assay, the colonies of Beta-HIVS treated group was fewer and smaller, and the colony numbers were significantly lower than those of two contrast groups ($P<0.01$) (Figure 1B).

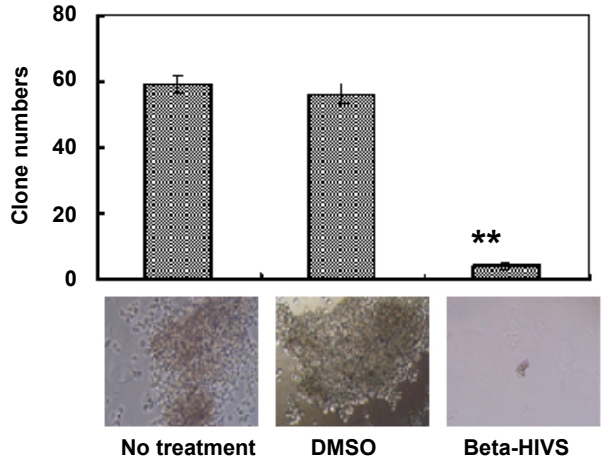

Figure 1b: Colony-forming assay. Cell colonies containing >50 cells were counted on day 14. Data shown are the means ± SD (n=15) (*: P<0.05; **: P<0.01, vs. no treatment cells).

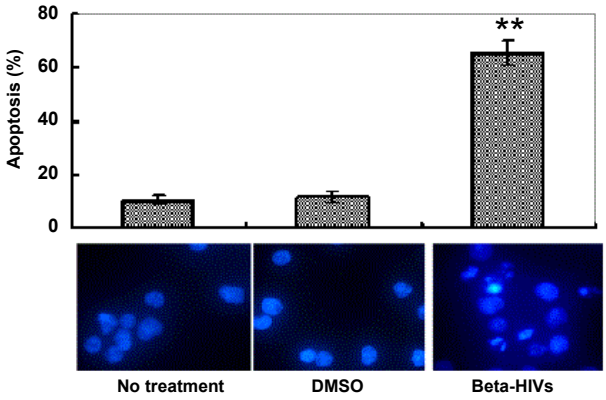

Figure 2a: Beta-HIVS promotes apoptosis in U266 multiple myeloma cells. DAPI fluorescence staining shows the microscopic changes in the nuclei of beta-HIVS treated cells observed (72h) via a fluorescence microscope, at x 400 magnification.The red arrow indicates typical morphology of the nucleus following apoptosis at phase IIb. (Non-viable apoptotic ratio) % = (phase IIb cell numbers/200 cells) x 100%. Data shown are the means ± SD (n=3) (**: P<0.01 vs. no treatment cells).

Apoptotic cell death was induced in U266 cells by beta-HIVS

The apoptosis-inducing effects were measured with the aid of DAPI staining and DNA ladder electrophoresis. As indicated in (Figure 2A), U266 cells in beta-HIVS group presented morphology of chromatin condensation and shrinkage in phase IIb of the apoptotic phase at 72 h following treatment, and the apoptotic ratio of phase IIb (%) was significantly higher than that of two control groups, with P<0.01. In contrast, cells of DMSO and no treatment groups all presented no marked apoptotic morphology. DNA degradative fragments were detected by DNA ladder electrophoresis, which also displayed typical apoptosis 'DNA ladders' in beta-HIVS treated group against that of two control groups (Figure 2B).

The intrinsic apoptotic pathway is involved in beta-HIVS induced cell apoptosis

Real-time PCR, immunofluorescence assay and western blotting were performed for the detection of apoptosis-related proteins, involved Bcl-2, Bax, PARP, cytochrome c, caspase-3 and caspase-9. Data of real-time PCR test evinced that the mRNA expression of *Bcl-2* in beta-HIVS treated group decreased, with P<0.01 vs. DMSO group, while the *Bax* mRNA expression changed unconspicuously (Figure 3A). At the same time, immunofluorescence analysis displayed that caspase-3 was activated in beta-HIVS treated group, accompanied by an increase in the levels of cytochrome c, as enhanced red fluorescence was observed in the beta-HIVS treated cells, which utilized anti-cleaved caspase-3 (Asp175) and anti-cytochrome c antibodies, combined by Cy3-conjugated anti-mouse or rabbit IgG. (Figure 3B). Similarly, western blotting showed that the expression of Bcl-2 in beta-HIVS

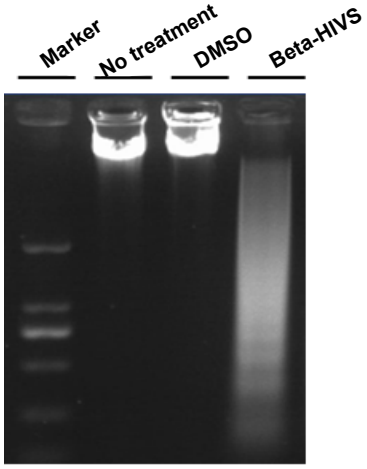

Figure 2b: DNA fragmentation analysis. DNA was extracted at 72 h following beta-HIVS treatment.

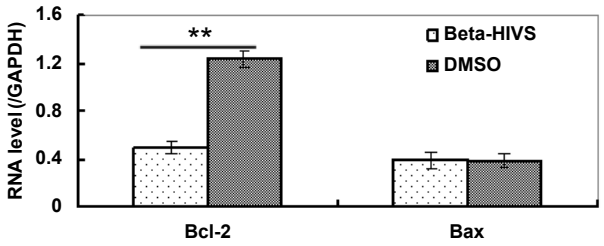

Figure 3a: Alterations of apoptosis-associated factors in different groups of U266 cells. Real-time PCR.

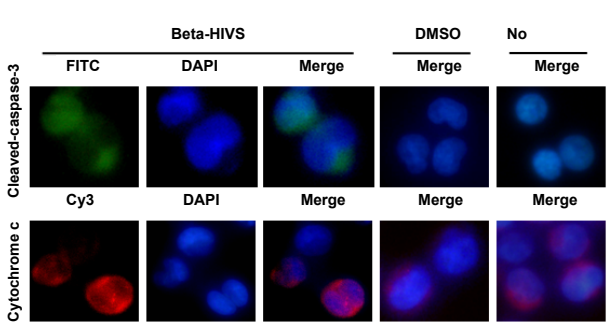

Figure 3b: Immunofluorescence staining, observed via a fluorescence microscope at 72 h after beta-HIVS treatment, at x 400 magnification. Cleaved caspase-3 was marked by green fluorescence (FITC), and cytochrome c was marked by red fluorescence (Cy3). The nucleus was stained by DAPI.

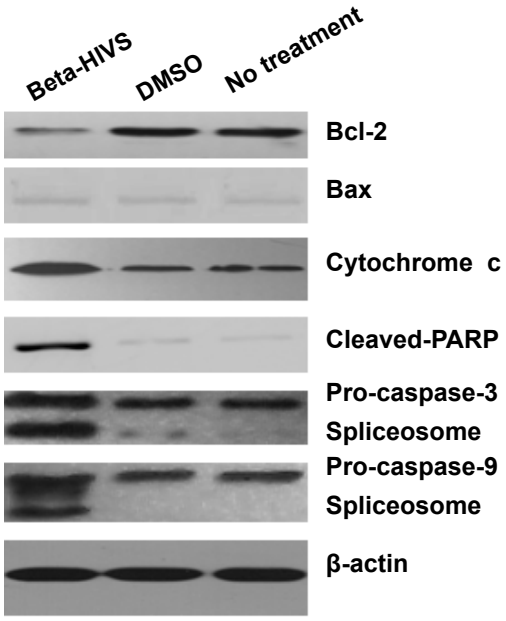

Figure 3c: Western blotting analysis of apoptosis-related proteins at 72 h after beta-HIVS treatment.

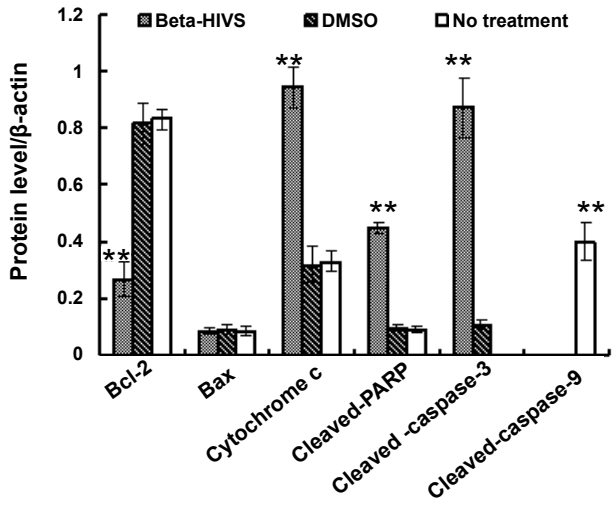

Figure 3d: Changes in expression of apoptosis-related proteins normalized to β-actin. Data shown are the means ± SD (n=3 or 5) (**: P<0.01 vs. no treatment cells).

group down-regulated, while cytochrome c expression up-regulated, all with P<0.01 when comparing the two contrast groups. Cleaved PARP was also detected in beta-HIVS treated group. Correspondingly, both cleaved caspase-3 and -9 were detected, as a 17 kDa spliceosome for pro-caspase-3 and a 37 kDa spliceosome for pro-caspase-9, were all observed in beta-HIVS treated group (Figure 3C and3D).

Discussion

In the present study, we first evaluated the apoptosis-inducing effect of beta-HIVS on U266 cells, with a result that this drug suppressed U266 cell proliferation and colony formation, and induced cell apoptosis. To further analyze the mechanism for beta-HIVS-induced apoptosis, we carried out real-time PCR, immunofluorescence staining and western blotting in the detection of changes of apoptosis-associated factors. Our data displayed that beta-HIVS treatment resulted in alterations of the expression of Bcl-2, cytochrome c, and an activation of PARP, caspase-3 and caspase-9 in U266 cells, indicating that beta-HIVS induces U266 cell death possibly by activating the mitochondria pathway.

Beta-HIVS is an ATP non-competitive inhibitor of protein-tyrosine kinases and is capable of inducing apoptosis in various lines of human tumor cells which has been documented by several publications [9-16]. Hashimoto et al, 2002 found that beta-HIVS was able to induce cell death more efficiently in DMS114 and NCI-H522 lung cancer cells [6]. Xu et al, 2004 also presented the similar results [18]. Masuda and his study group had previously validated the effects of beta-HIVS on U937 and HL-60 leukemia cells and found both two kinds of cell lines all suffered from apoptosis when treated with 10^{-6} M beta-HIVS [14]. Similarly, another proof from Hashimoto et al, 1999 also suggested that low concentrations of beta-HIVS between 10^{-8} and 10^{-6} M could inhibit the growth of various lines of cancer cells included HL-60 leukemia cells [15]. In consistant with the above findings, our data revealed that cell viability and colony formation of U266 cells were inhibited by beta-HIVS at a concentration of 1.2 μg/ml. Notably, the apoptosis-inducing effects were further validated by DAPI staining and DNA ladder electrophoresis assays, suggesting that beta-HIVS may be a potential therapeutic agent for MM treatment.

MM is considered the second most common hematological malignancy and is characterized by the clonal proliferation of neoplastic plasma cells in the bone marrow [1,2]. The improved understanding of the molecular mechanisms of MM development may provide a basis for the development of effective treatment strategies. Previous studies have demonstrated that beta-HIVS is one of the inhibitors of Protein Tyrosine Kinases (PTKs) which play important roles in a number of signal transduction pathways that are involved in cancer cell growth, differentiation, cell death, and carcinogenesis [19-22]. Particularly, beta-HIVS prohibits the activity of v-Src and EGFR in an ATP-non-competitive manner, and suppresses polo-like kinase 1 (PLK1) after inhibition of PTK activity, with resultant induction of apoptosis in human leukemia cells [23]. Correspondingly, a study from Xu et al, 2004 also indicated that beta-HIVS induced apoptosis in DMS114 cells via a PTK-dependent pathway [18]. Besides, Masuda et al, 2003 had demonstrated that beta-HIVS suppresses the growth of U937 and HL-60 leukemia cells by inhibiting the activity of a polo-like kinase 1 (PLK1) that is involved in control of the cell cycle [14].

In addition, other singling pathways are involved in beta-HIVS-mediated tumor cell death as well [15,24-27]. Early in 1999, Hashimoto et al. had reported that beta-HIVS was able to activate caspase-3 in its anti-neoplastic effect [15]. Then, Wu et al. described that shikonin inhibited cell growth in a time- and dose-dependent manner, accompanied by a decreased Bcl-2 and increased Bax expression, as well as an activation of caspase cascade [26]. Similarly, the present study showed that beta-HIVS treatment in U266 cells resulted in changes in the expression of Bcl-2, cytochrome c, PARP, caspase-3 and caspase-9, characterized by a reduced expression of Bcl-2 and increased expression of cytochrome c, although Bax expression altered with no significance. The activation of caspase-3 was further verified by western blotting and immunofluorescence assays. In addition, cleaved caspase-9 was detected by western blotting as well. Importantly, cleaved-PARP was also detected, which further provides evidence of the activation of caspase-3 and caspase-9. In short, it these data suggest that beta-HIVS suppresses cell viability and induces cell apoptosis by triggering the

mitochondrial pathway in multiple myeloma. Yet, our findings are in consistent with the previous studies [15,26,27].

Taken together, the present study disclosed the apoptosis-inducing of beta-HIVS on U266 cells as well as the primary underlying mechanisms. However, the evaluation of the apoptosis status of U266 cells induced by beta-HIVS was limited due to the detection methods conducted in this study, and therefore more apoptosis evaluations are needed. Moreover, the release of cytochrome c from the mitochondria should also be validated. These experiments will be performed in our future studies.

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