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Original Article

β-Hydroxyisovalerylshikonin promotes reactive oxygen species production in HCT116 colon cancer cells, leading to caspase-mediated apoptosis

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ABSTRACT

Although β-hydroxyisovalerylshikonin is suggested as a potential therapeutic agent for preventing various cancers, the underlying molecular mechanisms are not completely understood. In the present study, we investigated whether β-hydroxyisovalerylshikonin enhances apoptosis by triggering reactive oxygen species production in colon cancer HCT116 cells. β-Hydroxyisovalerylshikonin significantly inhibited the viability of HCT116 cells with maximum inhibition at 4 μM. Furthermore, treatment with β-hydroxyisovalerylshikonin subsequently increased sub-G_1 cells and annexin-V^+ cell population. Additionally, pretreatment with the caspase-8 inhibitor, z-IETD-fmk, and the caspase-9 inhibitor, z-LETD-fmk, significantly decreased β-hydroxyisovalerylshikonin-induced apoptosis, suggesting that β-hydroxyisovalerylshikonin promotes apoptosis through both the intrinsic and the extrinsic apoptotic pathways by activating caspase-8 and caspase-9. We also found that mitochondria played an important role in β-hydroxyisovalerylshikonin-mediated apoptosis via the intrinsic pathway. Accordingly, β-hydroxyisovalerylshikonin-induced reactive oxygen species production was evident after treatment with β-hydroxyisovalerylshikonin, and pretreatment with reactive oxygen species inhibitors, *N*-acetyl-L-cysteine and glutathione, significantly decreased β-hydroxyisovalerylshikonin-induced reactive oxygen species production, resulting in inhibition of apoptosis, which suggests that ROS generation is required for β-hydroxyisovalerylshikonin-mediated apoptosis. Taken together, these results demonstrated that the apoptotic effect of β-hydroxyisovalerylshikonin is enhanced in colon cancer HCT116 cells via reactive oxygen species generation and triggering of the caspase pathways, indicating that β-hydroxyisovalerylshikonin has potential as a therapeutic in the treatment of colon cancers.

Introduction

Reactive oxygen species (ROS) are natural byproducts of the normal metabolism of oxygen and play important roles in cell signaling, immunity, and homeostasis (West et al., 2011). ROS are classified into two groups; free-oxygen radicals and non-radical ROS. Free-oxygen radicals include superoxide ($O_2^{\bullet -}$), nitric oxide (NO•), hydroxyl radical (•OH), and organic radicals (R•), whereas hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and highly reactive lipid- or carbohydrate-derived carbonyl compounds are included in the non-radical ROS group (Birben et al., 2012). Normally, redox status is strongly balanced by the enzyme and non-enzyme systems (Wagener et al., 2013). However, redox balance is frequently disrupted by excessive ROS production and/or anti-oxidant depletion, leading to oxidative stress (Poljsak et al., 2013). In particular, aberrant ROS production is known as a potent mediator of inflammation, resulting in tissue injury and diseases such as cancer and neuronal disorder (Kehrer and Klotz, 2015). Thus, targeting ROS production is a promising therapeutic approach for inflammatory diseases and cancers. However, ROS also play an important role in apoptosis under both

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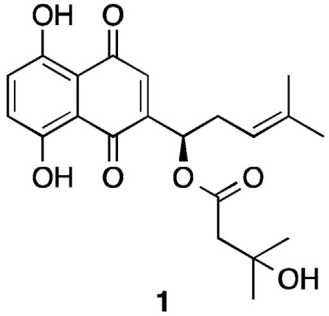
physiologic and pathologic conditions (Wagener et al., 2013). Enhanced production of intracellular ROS triggered apoptosis by activating the mitochondrial-dependent cell death pathway via stimulation of the mitogen-activated protein kinase (MAPK) pathways and proapoptotic signals, and thus subsequently stimulated mitochondrial membrane potentials, resulting in cell death (Li et al., 2011). Therefore, ROS are crucial messengers in determining cell death or cell survival.

Apoptosis was described in terms of characteristic changes in cell morphology, including cell shrinkage, chromatin condensation, nuclear fragmentation, and membrane blebbing (Indran et al., 2011). Apoptosis is implicated in a variety of biological processes, such as embryogenesis, regulation of the immune system, and elimination of damaged cells (Guicciardi et al., 2013). The importance of apoptosis has been emphasized by recent demonstrations involving various chemotherapeutic anti-cancer agents. Indeed, current anti-cancer therapy using many chemotherapeutic agents as well as ionizing radiation therapy activated the apoptotic machinery to kill cancer cells (Zhang et al., 2015). The last decade has shown an extraordinary development in investigation of apoptosis and cancer treatments by regulating the redox system (Circu and Aw, 2010). Furthermore, the molecular mechanisms that control and execute apoptotic cell death are being identified. In the future, it seems likely that rational strategies to manipulate cell apoptosis will be focused novel therapies that are more beneficial than current treatment regimens.

To date, a series of novel shikonin-derivative analogs bearing oxygen-containing substituents were investigated, among which, β-hydroxyisovalerylshikonin (**1**, HIVS) has been highlighted for exhibiting the strongest apoptosis-inducing activity. β-Hydroxyisovalerylshikonin isolated from traditional Asian species, *Lithospermum radix* (*Lithospermum erythrorhizon* Siebold & Zucc., Boraginaceae), induced apoptosis in various types of human cancer cells (Kajimoto et al., 2008; Komi et al., 2009). It was also reported that HIVS showed great promise as a potent apoptotic agent in human leukemia cells (Masuda et al., 2003). So far, only a few studies have reported that HIVS induced apoptosis in various cancer cell lines. Therefore, in this study, we investigated the apoptosis mechanism of HIVS regulation via ROS generation in colon cancer HCT116 cells.



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Materials and methods

Plant material and β-hydroxyisovalerylshikonin

The roots of *Lithospermum erythrorhizon* Siebold & Zucc., Boraginaceae, were purchased in Jecheon Market (Jecheon, Republic of Korea). A voucher specimen has been deposited in Wood Chemistry & Microbiology Department, Korea Forest Research Institute (Seoul, Republic of Korea). HIVS (**1**) was isolated and characterized in our previous study (Jayasooriya et al., 2014).

Antibodies and reagents

Antibodies against caspase-3, caspase-8, caspase-9, Bad, Bcl-2, Bid, poly(ADP-ribose) polymerase (PARP), cytochrome *c*, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A caspase-8 inhibitor, z-IETD-fmk, and a caspase-9 inhibitor, z-LETD-fmk, were purchased from Calbiochem (San Diego, CA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulins were purchased from KOMA Biotechnology (Seoul, Republic of Korea). 6-Carboxy-2′,7′-dichlorofluorescein diacetate (DCFDA) and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes (Eugene, OR). Glutathione (GSH), *N*-acetyl-L-cysteine (NAC), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO) and Roswell Park Memorial Institute Medium (RPMI), antibiotic mixture, and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea).

Cell line and growth assay

Human colon cancer HCT116 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI (WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% FBS and antibiotics (WelGENE Inc.) at 37 °C in a 5% CO_2-humidified incubator. The cells were seeded at 1×10^5 cells/ml and then treated with the indicated concentrations of HIVS for 24 h in the presence of various inhibitors. MTT assay was performed to determine relative cell viability.

DNA fragmentation

HCT116 cells were treated with various concentrations of HIVS for 24 h and then lysed on ice in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min. Lysates were vortexed and cleared by centrifugation at $10{,}000 \times g$ for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and analyzed electrophoretically on a 1.5% agarose gel containing ethidium bromide.

Flow cytometry analysis

HCT116 cells were treated with various concentrations of HIVS for 24 h in the presence of NAC and GSH. The cells (1×10^6) were fixed in 70% ethanol overnight at 4 °C and washed in phosphate-buffered saline (PBS) with 0.1% BSA. Then, the cells were incubated with 1 U/ml RNase A (DNase free) and 10 μg/ml propidium iodide (PI, Sigma) for 30 min in the dark. The level of apoptotic cells containing sub-G_1 DNA content was determined as a percentage of the total number of cells. For annexin-V staining, live cells were washed with PBS and then incubated with annexin-V fluorescein isothiocyanate (R&D Systems, Minneapolis, MN) for 30 min, until the cells were analyzed using flow cytometry. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used to determine the number of apoptotic cells, i.e., cells with sub-G_1 DNA that were annexin-V^+.

Western blot analysis

HCT116 cells were treated with various concentrations of HIVS for 24 h in the absence and the presence of NAC and GSH. The cells were lysed in buffer containing complete protease inhibitor mix (PRO-PREP) (iNtRON Biotechnology, Sungnam, Republic of Korea). After lysis for 30 min on ice, lysates were centrifuged at $14{,}000 \times g$

at 4 °C for 10 min. Supernatants were collected and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at −80 °C or immediately used for Western blot analysis. Proteins were blotted onto nitrocellulose membranes. The blots were then probed with 1:1000 dilution of antibodies followed by 1:10,000 dilution of goat anti-mouse horseradish peroxidase. Bands were imaged with an ECL reagent (Amersham, Arlington Heights, IL).

Caspase activity

The activity of caspase-like protease was measured using caspase activation kit according to manufacturer's protocol. This assay is based on spectrophotometric detection of the color reporter molecule *p*-nitroanaline (pNA) that is linked to the end of the caspase-specific substrate. The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantified spectrophotometrically at a wavelength of 405 nm. For the measurement of Ac-DEVD-pNA (for caspase-3), Ac-IETD-pNA (for caspase-8) and LEHD-pNA (for caspase-9) are used as the substrates.

Determination of mitochondrial membrane potential

The mitochondrial membrane potential was monitored by measuring the uptake of $DiOC_6$. Briefly, HCT116 cells were treated with various concentrations of HIVS for 24 h, loaded with 50 nM $DiOC_6$ at 37 °C for 30 min in the dark, and then analyzed using a flow cytometer.

Measurement of ROS

HCT116 cells were seeded on 24-well plate at a density of 1×10^5 cells/ml and preincubated with DCFDA for 1 h and then treated with the indicated concentrations of HIVS in the presence of GSH and NAC for 24 h. The cells were lysed with triton and the sample was centrifuged and supernatant was analyzed for ROS production using GLOMAX luminometer (Promega).

Statistical analysis

The images were visualized with Chemi-Smart 2000 (Vilber-Lourmat, Marine, Cedex, France). Images were captured using Chemi-Capt (VilberLourmat) and transported into Photoshop. All bands were shown a representative obtained in three independent experiments and quantified by Scion Imaging software (http://www.scioncorp.com). Statistical analyses were conducted using SigmaPlot software (version 12.0). Values were presented as mean ± standard error (SE). Significant differences between the groups were determined using the unpaired one-way and two-way ANOVA with Bonferroni's test. Statistical significance was regarded at $^{a,b}p < 0.05$.

Results

HIVS decreases the viability of colon cancer HCT116 cells

To assess whether HIVS affects the viability of colon cancer HCT116 cells, cytotoxic activity was analyzed by an MTT assay. Concentration-dependent treatment with HIVS gradually reduced the viability of HCT116 cells to 93.7 ± 3.1% at 0.5 μM, 72.3 ± 3.5% at 1.0 μM, 64.5 ± 5.8% at 2.0 μM and 47.4 ± 3.8% at 4.0 μM HIVS, respectively (Fig. 1A). As expected, HIVS significantly increased DNA fragmentation when the HIVS concentration rose (Fig. 1B). Distinct DNA ladders were observed from at 2 μM HIVS. To investigate in detail whether HIVS enhances apoptosis of HCT116 cells, we analyzed cell cycle distribution and total population of annexin-V^+ cells by flow cytometry analysis. HIVS increased the sub-G_1 cell populations to 1.5 ± 1.4% at 0.5 μM, 2.1 ± 1.8% at 1.0 μM, 4.9 ± 3.1% at 2.0 μM and 47.4 ± 3.8% at 4.0 μM HIVS (Fig. 2C, top), and annexin-V^+ cells by 24.5% (Fig. 1C, bottom). HIVS subsequently enhanced sub-G_1 and annexin-V^+ cell population in a concentration-dependent manner. In addition, a significant G_2/M phase arrest was observed by HIVS treatment. Taken together, these results indicate that HIVS promotes apoptosis of colon cancer HCT116 cells.

HIVS activates extrinsic and intrinsic apoptotic pathways

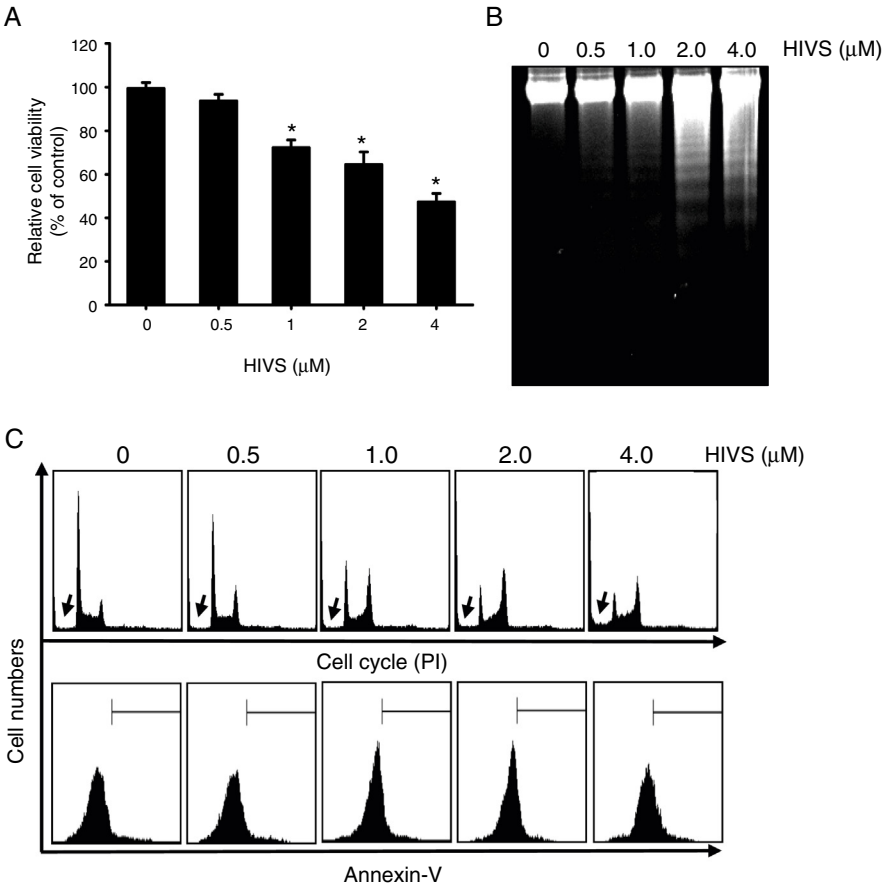
Because apoptosis is executed via different biochemical pathways by activating caspases (Ghobrial et al., 2005), we analyzed the expression and activity of caspases. HIVS decreased the proforms of caspase-3, caspase-8, and caspase-9 in HCT116 cells concomitant with the cleavage of PARP in a dose-dependent manner, implying that preforms are cleaved to form active caspases in response to HIVS (Fig. 2A). Next, cell lysates treated with HIVS were assayed for *in vitro* caspase activity. Treatment with HIVA greater than 2.0 μM significantly upregulated caspase-3, caspase-8 and caspase-9 (Fig. 2B). Finally, to examine the functional effect of caspases in HIVS-induced apoptosis, HCT116 cells were pretreated with a caspase-8 inhibitor, z-IETD-fmk, and a caspase-9 inhibitor, z-LETD-fmk, and then, the cells were exposed to HIVS for 24 h. Cell viability assay indicated that exposure to 4 μM HIVS decreased cell viability by approximately below 50% when compared with that of the untreated control (Fig. 2C and D). However, pretreatment with both z-IETD-fmk and z-LETD-fmk significantly restored the downregulated cell viability induced by HIVS at 24 h, suggesting that caspases play important roles in HIVS-mediated apoptosis. These data indicate that HIVS-induced apoptosis activates both the extrinsic and intrinsic pathways in HCT116 cells, leading to apoptosis.

HIVS regulates the expression of Bcl-2 family proteins and depolarizes mitochondrial membrane potential

We examined the effect of HIVS on the activation of the Bcl-2 protein family and mitochondrial membrane potential in the presence of HIVS. Western blot analysis showed that in HCT116 cells, HIVS substantially increased the expression of the pro-apoptotic Bad and decreased that of the anti-apoptotic Bcl-2, as well as truncated Bid (Fig. 3A). Additionally, we investigated whether HIVS regulates mitochondrial membrane potential. A marked decrease in the mitochondrial membrane potential occurred in HCT116 cells treated with HIVS in a dose-dependent manner (Fig. 3B). These data indicate that HIVS-induced intrinsic apoptosis passes through mitochondria accompanied by Bcl-2 family proteins.

ROS are involved in HIVS-induced apoptosis

To investigate whether ROS production is directly associated with HIVS-induced apoptosis, HCT116 cells were treated with the indicated concentrations of HIVS for 24 h and ROS production was measured using a luminometer. Treatment with HIVS markedly enhanced ROS production in HCT116 cells (Fig. 4A). Fluorometric data also confirmed that treatment with anti-oxidants such as GSH and NAC significantly attenuated the HIVS-induced ROS generation (Fig. 4B), resulting in increased cell viability (Fig. 4C). In detail, HIVS-induced annexin-V^+ cell population was significantly reduced in the presence of GSH and NAC (Fig. 4D), which indicates that ROS are key regulators in HIVS-mediated apoptosis. Additionally, Western blot analysis showed that HIVS-induced upregulation of cytosolic cytochrome *c* was significantly reduced by treatment with GSH and NAC (Fig. 4E). Moreover, phase-contrast microscopy showed



HIVS (μM)	Sub-G_1(%)	Annexin-V^+(%)
0	1.5±1.4	4.8±3.4
0.5	2.1±1.8	5.6±2.1
1.0	4.9±3.1	10.5±4.1
2.0	10.3±2.5*	15.7±2.3*
4.0	20.3±3.1*	26.7±4.8*

Fig. 1. Effect of HIVS on the viability of HCT116 colon cancer cells. HCT116 cells (1×10^5 cells/ml) were incubated with the indicated concentrations of HIVS (0–4 μM) for 24 h. (A) Cell viability was determined by MTT assay. (B) Total DNA was extracted from the cells and DNA fragmentation assay was performed using 1.5% agarose gel. (C) Cell cycle distribution (top) was analyzed by flow cytometry. In a parallel experiment, the percentage of apoptosis was analyzed by flow cytometry after annexin-V staining (bottom). Cell populations of sub-G_1 and annexin-V^+ are shown in the table. Data from three independent experiments are expressed as the overall mean ± SE. Statistical significance was determined by one-way ANOVA (*$p < 0.05$ vs. untreated control).

that HIVS-induced apoptotic cell shrinkage was attenuated by pre-treatment with GSH and NAC (Fig. 4F). These data indicate that HIVS-induced apoptosis is induced via ROS production in HCT116 cells.

Discussion

Owing to often-acquired drug resistance, treatment of colon cancer remains a challenge. Therefore, more effective and less toxic treatment alternatives or colon cancers would be of value (Kozovska et al., 2014). Recently, the utilization of naturally extracted bioactive compounds has received much attention for cancer chemoprevention. HIVS is a naturally derived shikonin analog and has been shown to exert various biological properties such as anti-inflammatory (Jayasooriya et al., 2014), anti-microbial (Damianakos et al., 2012), and anti-tumor effects (Hashimoto et al., 1999; Masuda et al., 2004). Our previous study reported that HIVS exhibited its anti-inflammatory activity via downregulation of proinflammatory mediators in BV2 microglial cells (Jayasooriya et al., 2014). Accordingly, a few studies reported the anti-cancer effect of HIVS. However, the detailed molecular action mechanisms of HIVS on human colon cancer cells have not been elucidated. The present study demonstrated that HIVS significantly increased ROS generation in colon cancer HCT116 cells, leading to apoptosis (Fig. 5).

Previous reports showed that HIVS promoted apoptosis in different cancer cells, including human leukemia cells (Hashimoto et al., 1999; Masuda et al., 2003), lung cancer cells (Hashimoto et al., 2002), and endometrial and ovarian cancer cells (Takai et al., 2008). Moreover, HIVS markedly diminished the growth of U937 human lymphoma cells, VMRC-MELG malignant melanoma cells, COLO320DM colorectal adenocarcinoma cells, AZ-521 gastric cancer cells, and MIA Paca-2 pancreatic cancer cells (Hashimoto et al., 1999). Nevertheless, the molecular action of HIVS is not

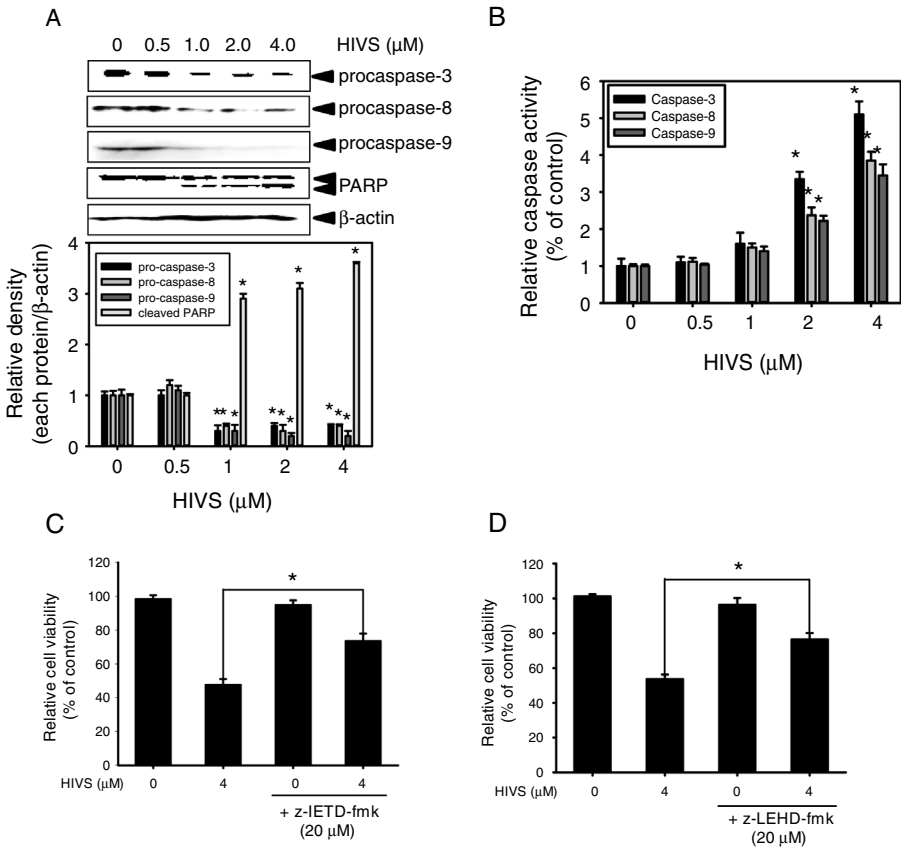
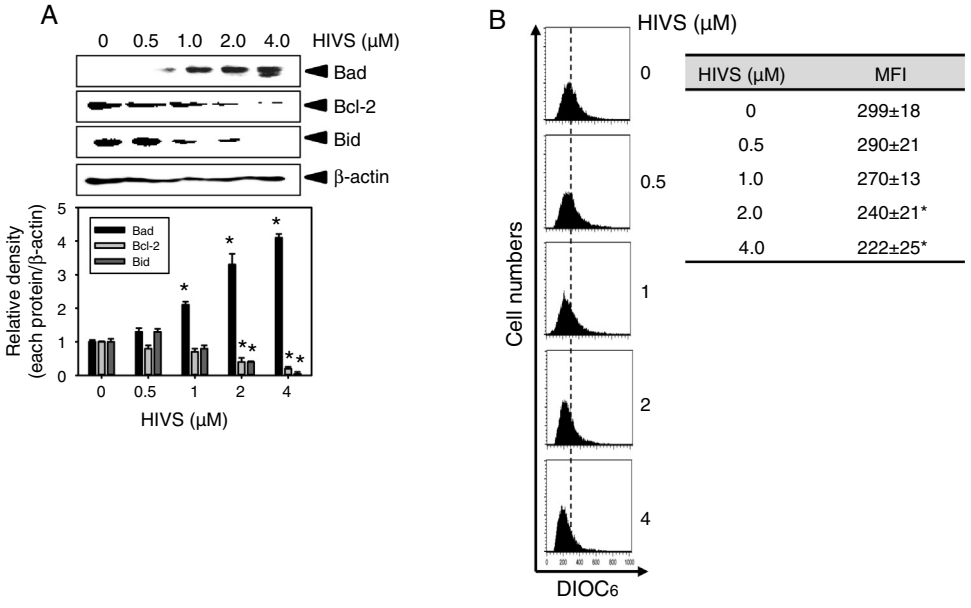


Fig. 2. HIVS-induced intrinsic and extrinsic apoptotic pathways in HCT116 colon cancer cells. HCT116 cells were seeded at a density of 1×10^5 cells/ml and incubated with the indicated concentrations of HIVS for 24 h. (A) Total cell lysates were prepared and aliquots containing 30 μg of protein were subjected to SDS-PAGE followed by western blot analysis with specific antibodies against procaspase-3, procaspase-8, procaspase-9, and PARP. β-Actin was used as an internal loading control. (B) Caspase activities were determined following the manufacturer's protocol. (C, D) In a parallel experiment, HCT116 cells were seeded at a density of 1×10^5 cells/ml and incubated with HIVS (4 μM) after treatment with z-IEHD-fmk (20 μM) and z-LETD-fmk (20 μM) for 2 h. Cell viability was determined by MTT assay at 24 h. Data from three independent experiments are expressed as the overall mean ± SE. Statistical significance was determined by one-way ANOVA [$^*p < 0.05$ vs. untreated control (A, B) and HIVS-treated group (C, D)].



HIVS (μM)	MFI
0	299±18
0.5	290±21
1.0	270±13
2.0	240±21*
4.0	222±25*

Fig. 3. Regulation of Bcl family and mitochondrial depolarization by HIVS. HCT116 cells were seeded at a density of 1×10^5 cells/ml and incubated with the indicated concentrations of HIVS for 24 h. (A) Total cell lysates were prepared and aliquots containing 30 μg of protein were subjected to SDS-PAGE followed by western blot analysis with specific antibodies against Bad, Bcl-2, and Bid. β-Actin was used as an internal loading control. (B) In a parallel experiment, mitochondrial potential was measured using $DiOC_6$ dye. Data from three independent experiments are expressed as the overall mean ± SE. Statistical significance was determined by one-way ANOVA ($^*p < 0.05$ vs. untreated control).

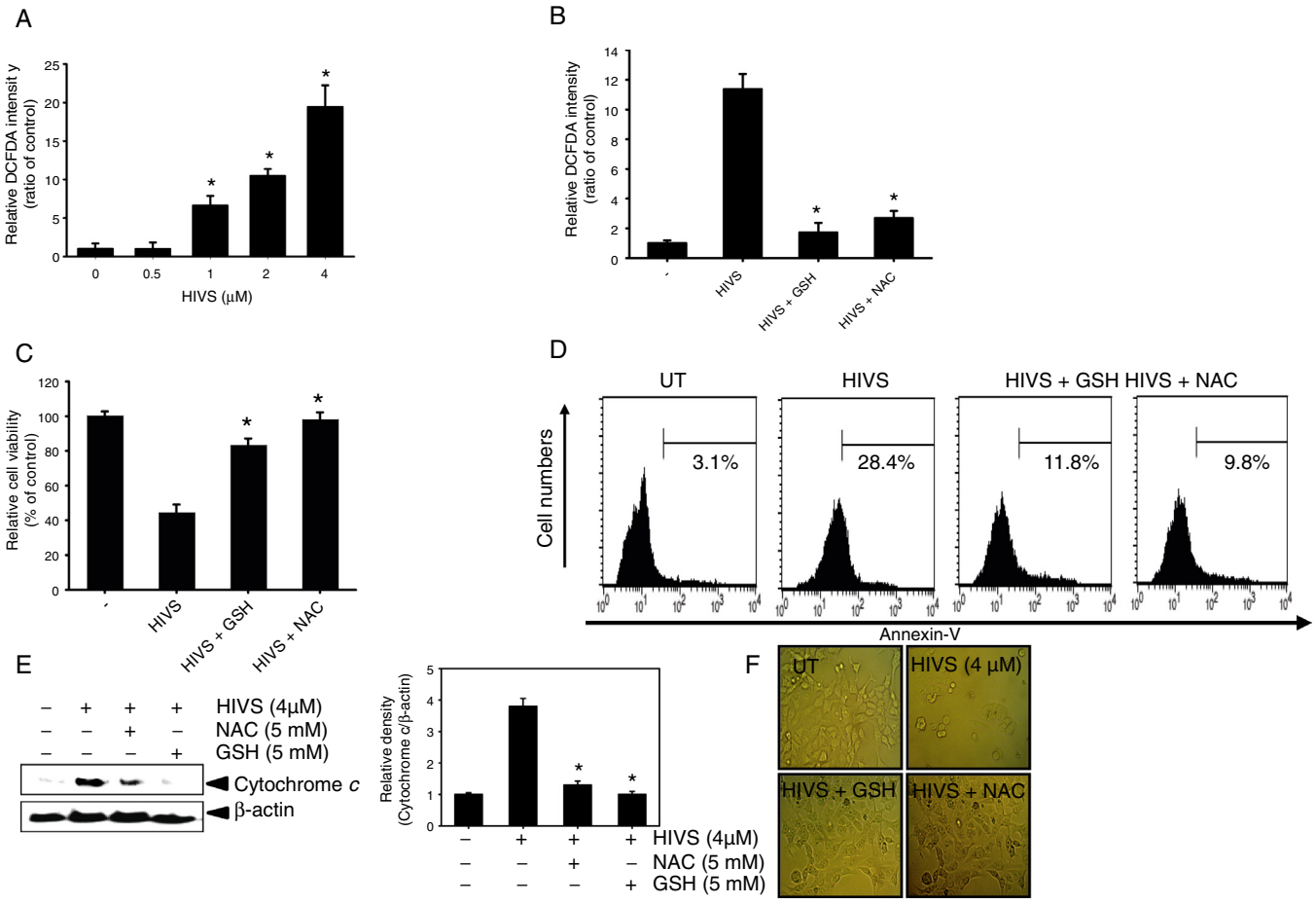


Fig. 4. HIVS-induced ROS production. (A) HCT116 cells were seeded at a density of 1×10^5 cells/ml, incubated with 20 μM DCFDA for 1 h, and then treated with 4 μM HIVS for 24 h. The cells were lysed and centrifuged at 16,000 × *g* for 10 min. Then, cell supernatant was collected and ROS production was analyzed by fluorometry. Relative ROS production was presented. (B, C) HIVS (4 μM) was treated in the presence of GSH (5 mM) and NAC (5 mM) for 24 h, and ROS production (B) and cell viability (C) were analyzed by fluorometry and MTT assay, respectively. (D) The percentage of apoptosis was analyzed by flow cytometry after annexin-V staining. Cell populations of annexin-V$^+$ are shown in each panel. (E) Cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed using antibodies. β-Actin was used as a loading control. (F) The morphological changes of HCT116 cells were observed by light microscopy (400×). Data from three independent experiments are expressed as the overall mean ± SE. Statistical significance was determined by one-way ANOVA [$^*p < 0.05$ vs. untreated control (A) and HIVS-treated group (C–E)].

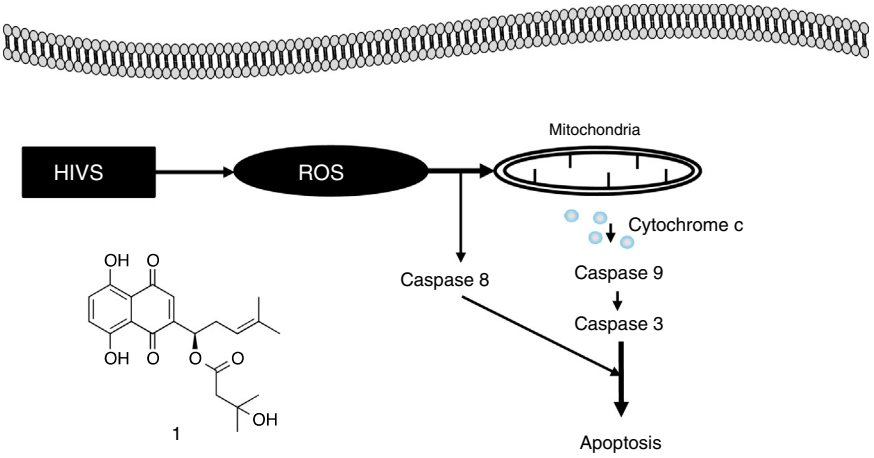


Fig. 5. Proposed hypothesis for HIVS-induced apoptosis. HIVS stimulates ROS generation, which consequently trigger the intrinsic and the extrinsic apoptotic pathway.

completely understood. HIVS triggered apoptosis in lung cancer cells by inhibiting protein tyrosine kinase (PTK) activity (Hashimoto et al., 2002). Kajimoto et al. (2008) showed that HIVS enhanced apoptosis of human lung cancer DMS114 cells via downregulation of dUTP nucleotidohydrolase activity. Moreover, the synergistic apoptotic effect of HIVS with cisplastin was reported against human lung cancer cells (Xu et al., 2004). Interestingly, in the present study, we found that colon cancer HCT116 cells treated with 4 μM HIVS for 24 h showed significant reduction of cell viability of approximately 50%, whereas the percentage of sub-G_1 phase and annexin-V^+ cells was approximately 20% each, suggesting that HIVS gently enhances apoptosis, resulting from the induction of cell stagnation or weak G_2/M phase arrest. Nishida et al. (2006) previously found that HIVS induced G_0/G_1 cell cycle arrest and apoptosis of endometriotic stromal cells, indicating the differential effects of HIVS against various cancers (Nishida et al., 2006).

Numerous reports showed that shikonin induced apoptosis via generation of ROS in various types of cancer cell lines (Ahn et al., 2013; Duan et al., 2014) Nevertheless, whether HIVS regulates ROS production has not been studied. Therefore, we hypothesized that HIVS-induced apoptosis resulted from ROS production in HCT116 cells. HIVS-induced ROS generation was evident and pretreatment with GSH and NAC significantly decreased the HIVS-induced ROS level, resulting in restoration of cell survival. These results indicate that ROS act as upstream signaling molecules to initiate HIVS-mediated apoptosis. As previously known, ROS play a critical role in controlling mitogen-activated protein kinases (MAPK) signaling, thus promoting apoptosis in many cancer cells by shikonin (Ahn et al., 2013; Huang et al., 2014). Therefore, further studies are required to clarify which protein kinases are regulated by HIVS in ROS-meditated apoptosis.

It is known that two key molecular signaling pathways govern apoptotic cell death (Ghobrial et al., 2005). The first is the intrinsic pathway, which is activated from inside the cell by members of the Bcl-2 family, resulting from mitochondrial signals, whereas the extrinsic pathway is activated by pro-apoptotic ligands (Ashkenazi, 2015). However, both pathways are intricately connected and activate an identical downstream caspase cascade, which carries out numerous proteolytic cleavages that enhance apoptosis (Ghobrial et al., 2005). Our findings indicated that treatment with HIVS activated both the intrinsic and the extrinsic apoptotic pathways. Caspase-8 activity, enhanced by HIVS, increased apoptosis and proved activation of the extrinsic pathway; pretreatment with a caspase-8 inhibitor, z-IETD-fmk, significantly inhibited HIVS-induced apoptosis. Accordingly, activation of caspase-9 enhanced HIVS-induced apoptosis by stimulating the intrinsic pathway. Pretreatment with a caspase-9 inhibitor, z-LETD-fmk substantially attenuated HIVS-induced apoptosis in HCT116 cells. Taken together, these data indicate that external and mitochondrial signals play a central role in initiation of HIVS-mediated apoptosis. Subsequently, many reports state that mitochondria are key regulators of both ROS production and the expression of apoptotic protein in the initiation and progression of various cancer cells. Therefore, mitochondria-targeted compounds have been a promising strategy in pre-clinical and early clinical trials (Modica-Napolitano and Weissig, 2015). Given our results, the present study demonstrated that HIVS potentiated the triggering of the apoptotic cascade via the intrinsic and the extrinsic pathways by stimulating ROS generation in HCT116 colon cancer cells.

Authors' contribution

MGD, WAHMK and IMNM took part in the evaluation of MTT assay, DNA fragmentation assay, western blot analysis and interpretation of data. CHK and JWJ carried out flow cytometry and fluorometic analysis. YHC and GYK contributed to all experimental designs. All the authors have contributed to critical reading and comments, and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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References

Ahn, J., Won, M., Choi, J.H., Kim, Y.S., Jung, C.R., Im, D.S., Kyun, M.L., Lee, K., Song, K.B., Chung, K.S., 2013. Reactive oxygen species-mediated activation of the Akt/ASK1/p38 signaling cascade and p21 (Cip1) downregulation are required for shikonin-induced apoptosis. Apoptosis 18, 870–881.

Ashkenazi, A., 2015. Targeting the extrinsic apoptotic pathway in cancer: lessons learned and future directions. J. Clin. Invest. 125, 487–489.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organ. J. 5, 9–19.

Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic. Biol. Med. 48, 749–762.

Damianakos, H., Kretschmer, N., Syklowska-Baranek, K., Pietrosiuk, A., Bauer, R., Chinou, I., 2012. Antimicrobial and cytotoxic isohexenylnaphthazarins from *Arnebia euchroma* (Royle) Jonst. (Boraginaceae) callus and cell suspension culture. Molecules 17, 14310–14322.

Duan, D., Zhang, B., Yao, J., Liu, Y., Fang, J., 2014. Shikonin targets cytosolic thioredoxin reductase to induce ROS-mediated apoptosis in human promyelocytic leukemia HL-60 cells. Free Radic. Biol. Med. 70, 182–193.

Ghobrial, I.M., Witzig, T.E., Adjei, A.A., 2005. Targeting apoptosis pathways in cancer therapy. CA Cancer J. Clin. 55, 178–194.

Guicciardi, M.E., Malhi, H., Mott, J.L., Gores, G.J., 2013. Apoptosis and necrosis in the liver. Compr. Physiol. 3, 977–1010.

Hashimoto, S., Xu, M., Masuda, Y., Aiuchi, T., Nakajo, S., Cao, J., Miyakoshi, M., Ida, Y., Nakaya, K., 1999. β-Hydroxyisovalerylshikonin inhibits the cell growth of various cancer cell lines and induces apoptosis in leukemia HL-60 cells through a mechanism different from those of Fas and etoposide. J. Biochem. 125, 17–23.

Hashimoto, S., Xu, Y., Masuda, Y., Aiuchi, T., Nakajo, S., Uehara, Y., Shibuya, M., Yamori, T., Nakaya, K., 2002. β-Hydroxyisovalerylshikonin is a novel and potent inhibitor of protein tyrosine kinases. Jpn. J. Cancer Res. 93, 944–951.

Huang, W.R., Zhang, Y., Tang, X., 2014. Shikonin inhibits the proliferation of human lens epithelial cells by inducing apoptosis through ROS and caspase-dependent pathway. Molecules 19, 7785–7797.

Indran, I.R., Tufo, G., Pervaiz, S., Brenner, C., 2011. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. Biochim. Biophys. Acta 1807, 735–745.

Jayasooriya, R.G., Lee, K.T., Lee, H.J., Choi, Y.H., Jeong, J.W., Kim, G.Y., 2014. Anti-inflammatory effects of β-hydroxyisovalerylshikonin in BV2 microglia are mediated through suppression of the PI3K/Akt/NF-κB pathway and activation of the Nrf2/HO-1 pathway. Food Chem. Toxicol. 65, 82–89.

Kajimoto, S., Horie, M., Manabe, H., Masuda, Y., Shibayama-Imazu, T., Nakajo, S., Gong, X.F., Obama, T., Itabe, H., Kakaya, K., 2008. A tyrosine kinase inhibitor, β-hydroxyisovalerylshikonin, induced apoptosis in human lung cancer DMS114 cells through reduction of dUTP nucleotidohydrolase activity. Biochim. Biophys. Acta 1782, 41–50.

Kehrer, J.P., Klotz, L.O., 2015. Free radicals and related reactive species as mediators of tissue injury and disease: implications for health. Crit. Rev. Toxicol. 45, 765–798.

Komi, Y., Suzuki, Y., Shimamura, M., Kajimoto, S., Nakajo, S., Masuda, M., Shibuya, M., Itabe, H., Shimokado, K., Oettgen, P., Nakaya, K., Kojima, S., 2009. Mechanism of inhibition of tumor angiogenesis by β-hydroxyisovalerylshikonin. Cancer Sci. 100, 269–277.

Kozovska, Z., Gabrisova, V., Kucerova, L., 2014. Colon cancer: cancer stem cells markers, drug resistance and treatment. Biomed. Pharmacother. 68, 911–916.

Li, Z.Y., Yang, Y., Ming, M., Liu, B., 2011. Mitochondrial ROS generation for regulation of autophagic pathways in cancer. Biochem. Biophys. Res. Commun. 414, 5–8.

Masuda, Y., Nishida, A., Hori, K., Hirabayashi, T., Kajimoto, S., Nakajo, S., Kondo, T., Aska, M., Nakaya, K., 2003. β-Hydroxyisovalerylshikonin induces apoptosis in human leukemia cells by inhibiting the activity of a polo-like kinase 1 (PLK1). Oncogene 22, 1012–1023.

Masuda, Y., Shima, G., Aiuchi, T., Horie, M., Hori, K., Nakajo, S., Kajimoto, S., Shibayama-Imazu, T., Nakaya, K., 2004. Involvement of tumor necrosis factor receptor-associated protein 1 (TRAP1) in apoptosis induced by β-hydroxyisovalerylshikonin. J. Biol. Chem. 279, 42503–42515.

Modica-Napolitano, J.S., Weissig, V., 2015. Treatment strategies that enhance the efficacy and selectivity of mitochondria-targeted anticancer agents. Int. J. Mol. Sci. 16, 17394–17421.

Nishida, M., Nasu, K., Ueda, T., Yuge, A., Takai, N., Narahara, H., 2006. β-Hydroxyisovalerylshikonin induces apoptosis and G0/G1 cell-cycle arrest of

endometriotic stromal cells: a preliminary in vitro study. Hum. Reprod. 21, 2850–2856.

Poljsak, B., Šuput, D., Milisav, I., 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. Oxid. Med. Cell Longev., http://dx.doi.org/10.1155/2013/956792.

Takai, N., Ueda, T., Nishida, M., Nasu, K., Narahara, H., 2008. β-Hydroxyisovalerylshikonin has a profound anti-growth activity in human endometrial and ovarian cancer cells. Gynecol. Oncol. 109, 107–114.

Wagener, F.A., Carels, C.E., Lundvig, D.M., 2013. Targeting the redox balance in inflammatory skin conditions. Int. J. Mol. Sci. 14, 9126–9167.

West, A.P., Shadel, G.S., Ghosh, S., 2011. Mitochondria in innate immune responses. Nat. Rev. Immunol. 11, 389–402.

Xu, Y., Kajimoto, S., Nakajo, S., Nakaya, K., 2004. β-Hydroxyisovalerylshikonin and cisplatin act synergistically to inhibit growth and to induce apoptosis of human lung cancer DMS114 cells via a tyrosine kinase-dependent pathway. Oncology 66, 67–75.

Zhang, L., Wang, K., Lei, Y., Li, Q., Nice, E.C., Huang, C., 2015. Redox signaling: potential arbitrator of autophagy an apoptosis in therapeutic response. Free Radic. Biol. Med. 89, 452–465.