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Reevesioside F induces potent and efficient anti-proliferative and apoptotic activities through Na⁺/K⁺-ATPase α_3 subunit-involved mitochondrial stress and amplification of caspase cascades

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Abstract

Reevesioside F, isolated from *Reevesia formosana*, induced anti-proliferative activity that was highly correlated with the expression of Na⁺/K⁺-ATPase α_3 subunit in several cell lines, including human leukemia HL-60 and Jurkat cells, and some other cell lines. Knockdown of α_3 subunit significantly inhibited cell apoptosis suggesting a crucial role of the α_3 subunit. Reevesioside F induced a rapid down-regulation of survivin protein, followed by release of cytochrome *c* from mitochondria and loss of mitochondrial membrane potential (Ψ_m). Further examination demonstrated the mitochondrial damage in leukemic cells through Mcl-1 down-regulation, Noxa up-regulation and an increase of the formation of truncated Bid, tBim and a 23-kDa cleaved Bcl-2 fragment. Furthermore, reevesioside F induced an increase of mitochondria-associated acetyl α -tubulin that may also contribute to apoptosis. The caspase cascade was profoundly activated by reevesioside F. Notably, the specific caspase-3 inhibitor z-DEVD-fmk significantly blunted reevesioside F-induced loss of Ψ_m and apoptosis, suggesting that caspase-3 activation may further amplify mitochondrial damage and apoptotic signaling cascade. In spite of being a cardiac glycoside, reevesioside F did not increase the intracellular Ca²⁺ levels. Moreover, CGP-37157 which blocked Na⁺/Ca²⁺ exchanger on plasma membrane and mitochondria did not modify reevesioside F-mediated effect. In summary, the data suggest that reevesioside F induces apoptosis through the down-regulation of survivin and Mcl-1, and the formation of pro-apoptotic fragments from Bcl-2 family members. The loss of Ψ_m and mitochondrial damage are responsible for the activation of caspases. Moreover, the amplification of caspase-3-mediated signaling pathway contributes largely to the execution of apoptosis in leukemic cells.

Keywords

Reevesioside F; Na⁺/K⁺-ATPase α_3 subunit; Survivin; Mitochondrial damage; Bcl-2 family of protein

1. Introduction

Several members of cardiac glycosides, a class of naturally derived compounds that bind to and block Na⁺/K⁺-ATPase, have long been in clinical therapy for the treatment of atrial arrhythmia and heart failure [1,2]. Recently, the inhibitors of Na⁺/K⁺-ATPase have attracted much interest in anticancer research. A variety of human cancers have been found to differentially express varying levels of Na⁺/K⁺-ATPase subunit isoforms. Certain isoforms have been demonstrated to be up-regulated in specific cancers [3–5]. Moreover, numerous findings reveal that particular Na⁺/K⁺-ATPase isoforms may be a marker for the progression of epithelial-to-mesenchymal transition (EMT) in cancers [6], suggesting that Na⁺/K⁺-ATPase can be a target for anticancer therapy. Consistent to the notion, a lot of preclinical data demonstrate that cardiac glycosides display anti-proliferative as well as apoptotic activities in various cancers, including prostate, breast, lung, leukemia and colorectal cancers [2–4,7,8]. Furthermore, cardiac glycoside-based anticancer drugs are currently under clinical trials [2,9].

Cardiac glycosides induce anticancer activity through various mechanisms, including an increase of intracellular Ca²⁺ levels, up-regulation of death receptors, down-regulation of survivin, production of reactive oxygen species (ROS), induction of p21^{Cip1} and inhibition of hypoxia-inducible factor 1 α synthesis [10–13]. It is noteworthy that although some of the mechanisms are Ca²⁺-dependent activities that are attributed to the inhibition of Na⁺/K⁺-ATPase, cardiac glycosides can also regulate numerous cellular processes via mechanisms that are beyond the well-established role in ion homeostasis [2]. The anti-leukemic activities of cardiac glycosides have also been documented through a blockade of nuclear factor- κ B (NF- κ B) signaling pathway, resulting in decreased expression levels of pro-survival proteins, induction of mitochondrial stress and activation of caspase cascades [8,14]. Although cardiac glycosides show anticancer potential, the therapeutic window is limited by the cardiotoxic effects, including arrhythmogenic effects [15]. However, the toxic effects are dependent on the dosage, the characters of the cardiac glycosides and the selectivity between cancer cells and cardiac cells. It is, therefore, anticipated that novel chemotherapeutics specifically targeting leukemic cells can be successfully developed.

Recently, the bioassay-guided fractionation of the root of *Reevesia formosana* led to the isolation of new cardiac glycosides in our work [16]. The present study showed that reevesioside F displayed potent activity against several leukemia cell lines, including HL-60 (human promyelocytic leukemia cells), CCRF-CEM (human acute lymphocytic leukemia cells) and Jurkat (human acute lymphocytic leukemia cells). Therefore, the anti-proliferative and apoptotic signaling pathways of reevesioside F have been identified.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Antibodies to GAPDH, Na⁺/K⁺-ATPase α_3 subunit, Bcl-2, Bid, Bak, Bax, Mcl-1, Noxa, Bim, cytochrome *c* and anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to α -tubulin, poly(ADP-ribose)polymerase (PARP), acetyl histone H3^{Lys9}, γ -H2A.X, calnexin and caspase-3, -7, -8 and -9 were from Cell Signaling Technologies (Boston, MA). Methylthiazolyldiphenyl-tetrazolium bromide (MTT), propidium iodide (PI), phenylmethylsulfonylfluoride (PMSF), leupeptin, dithiothreitol (DTT), Triton X-100, RNase, aprotinin, sodium orthovanadate and all of the other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO). Reevesioside F (Fig. 1A) was isolated from the root of *Reevesia formosana*. The purification and identification of reevesioside F were published elsewhere [16].

2.2. Cell lines and cell culture

HL-60 (promyelocytic leukemia) and Jurkat (T-cell acute lymphoblastic leukemia) were from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium with 10% FBS (v/v) and penicillin (100 U/ml)/streptomycin (100 μ g/ml). Cultures were maintained in a humidified incubator at 37 °C in 5% CO₂/95% air.

2.3. Mitochondrial MTT reduction activity assay

Cells were incubated in the absence or presence of the compound for the indicated concentrations and times. After the treatment, the mitochondrial MTT reduction activity was assessed. MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/ml and filtered. From the stock solution, 10 μ l per 100 μ l of medium was added to each well, and plates were gently shaken and incubated at 37 °C for 2 h. After the loading of MTT, the medium was replaced with 100 μ l acidified β -isopropanol and was left for 5–10 min at room temperature for color development. The 96-well plate was read by enzyme-linked immunosorbent assay reader (570 nm) to get the absorbance density values.

2.4. Flow cytometric assay of DNA content

After the treatment of cells with the indicated agent, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4 °C for 30 min and washed with PBS. After centrifugation, cells were incubated in 0.1 ml of phosphate-citric acid buffer (0.2 M NaHPO₄, 0.1 M citric acid, pH7.8) for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 ml PI solution containing Triton X-100 (0.1% v/v), RNase (100 μ g/ml) and PI (80 μ g/ml). DNA content was analyzed with FACScan and CellQuest software (Becton Dickinson, Mountain View, CA).

2.5. DNA fragmentation assay

After the treatment, cells were collected in a buffer containing 20 mM Tris pH 7.0 and 250 mM sucrose on ice. Total DNA was extracted by Genomic DNA kits (Geneaid, Taiwan).

DNA was subsequently subjected to electrophoresis on 2% agarose gels containing SYBR[®] green I (1:250 dilution of stock in TE buffer) (Molecular Probes, Eugene, OR), and visualized under UV light.

2.6. Confocal microscopic examination with DAPI staining

After the treatment, the cells were fixed with 100% methanol at -20°C for 5 min and incubated in 1 $\mu\text{g}/\text{ml}$ DAPI for nuclear staining, or in the indicated antibodies for the detection of specific proteins. The cells were analyzed by a confocal laser microscopic system (Leica TCS SP2).

2.7. Microscopic observation of cell morphology

After the treatment, cells were collected by centrifugation, resuspended in 200 μl of PreserveCyt solution (PBS plus methanol). The suspension was passed through a Thinprep processing machine, and the cells were collected. The slides were fixed in 95% alcohol and then stained with Wright-Giemsa for 5 min at room temperature. Stained cells from each treatment group were examined under an Olympus fluorescence microscope.

2.8. Transfection of HL-60 cells with α_3 subunit siRNA

Protein expression in HL-60 cells was induced using electroporation. Cells were resuspended in 250 μl of BTXpress Solution (BTX Harvard Apparatus, Holliston, MA). Cells were placed in an Eppendorf tube and α_3 silencing RNA (50 nM; Santa Cruz Biotechnology) was added; cells were then transferred into a 4-mm gap cuvette and electroporated with a BTX ECM 830 using a single pulse at 275 V, 950 μF for 14 ms. Cuvette contents were transferred to a six-well plate, media was removed and replaced. Twenty-four hours after transfection, cells were treated with 100 nM of reevesioside F for 24 h. The cells were harvested for flow cytometric analysis of PI staining or the protein was collected for Western blot analysis.

2.9. Measurement of mitochondrial membrane potential (Ψ_m)

JC-1, a mitochondrial dye staining mitochondria in living cells in a membrane potential-dependent fashion, was used to determine Ψ_m . Cells were treated in the absence or presence of the indicated agent. Thirty minutes before the termination of incubation, the cells were incubated with JC-1 (final concentration of 2 μM) at 37°C for 30 min. The cells were finally harvested and the accumulation of JC-1 was determined using flow cytometric analysis.

2.10. Western blotting

After the treatment, cells were harvested with trypsinization, centrifuged and lysed in 0.1 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 50 mM NaF and 100 μM sodium orthovanadate. In some experiments, the mitochondrial/cytosol fractionation kit (Biovision, Mountain View, CA) was used to separate mitochondrial and cytosolic fraction. Total protein was quantified, mixed with sample buffer and boiled at 90°C for 5 min. Equal amount of protein (30 μg) was separated by electrophoresis in 12% SDS-PAGE, transferred

to PVDF membranes and detected with specific antibodies. The immunoreactive proteins after incubation with appropriately labeled secondary antibody were detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

2.11. Measurement of intracellular Ca^{2+} level

After incubation with fluo-3/AM (5 μM) for 30 min, cells were washed twice and incubated in fresh medium. Vehicle (0.1% DMSO) or reevesioside F was added to cells and intracellular Ca^{2+} level was measured by flow cytometric analysis.

2.12. Data analysis

Data are presented as the mean $\pm$ SEM for the indicated number of separate experiments. Statistical analysis of data for multiple groups is performed with one-way analysis of variance (ANOVA). Student's *t*-test is applied for comparison of two groups. *P*-values less than 0.05 are statistically considered significant.

3. Results

3.1. Reevesioside F induces anti-proliferative and apoptotic activity

HL-60 (human promyelocytic leukemia cells) and Jurkat (human acute lymphocytic leukemia cells) were used to determine the anti-proliferative and apoptotic activity of reevesioside F. The data by MTT assay showed that reevesioside F induced a potent inhibition of cell survival with IC_{50} values in ten nanomolar range (Fig. 1B). Flow cytometric analysis of DNA staining showed that reevesioside F induced a time-dependent increase of hypodiploid (sub-G1) DNA content (Fig. 1C). The programmed cell death was also examined using DNA fragmentation assay (Fig. 1D) and microscopic examination of DAPI and Wright-Giemsa staining (Fig. 1E and F). The data revealed that reevesioside F induced a potent apoptotic activity in leukemic cells.

3.2. Levels of Na^+/K^+ ATPase α_3 subunit correlate well with reevesioside F-induced activity

Cardiac glycosides have been documented to bind to and block Na^+/K^+ -ATPase, leading to the accumulation of intracellular Na^+ which *in turn* triggers an increase of intracellular Ca^{2+} levels through the activation of $\text{Na}^+/\text{Ca}^{2+}$ exchanging system. In addition to ion pumping activity, certain subunit of Na^+/K^+ -ATPase has been reported to conduct non-pumping functions [3,5]. After the examination of protein expression of Na^+/K^+ -ATPase subunits and anti-proliferative activity of reevesioside F in several cancer cell lines, the data revealed that the expression levels of α_3 subunit highly correlated with the anti-proliferative activity (Fig. 2A). The knockdown of Na^+/K^+ -ATPase α_3 subunit significantly rescued reevesioside F-induced apoptosis and the cleavage of caspase-3 (Fig. 2B and C). However, reevesioside F did not modify the intracellular Ca^{2+} concentration using flow cytometric analysis of fluo-3 staining (Fig. 3A). Further functional identification using the specific inhibitor CGP-37157, which blocks $\text{Na}^+/\text{Ca}^{2+}$ exchanger on plasma membrane and mitochondria, showed that CGP-37157 did not inhibit reevesioside F-mediated effects, including the loss of ψ_m (Fig. 3B) and apoptotic cell death (Fig. 3C). The data suggested that reevesioside F induced the apoptotic effect through the signaling pathway independent of intracellular Ca^{2+} mobilization.

3.3. Reevesioside F regulates the expression of Bcl-2 family proteins and induces the loss of mitochondrial membrane potential

Mitochondria, the major source supplying cellular energy, are involved in numerous cellular functions, including differentiation, cell growth, cell death and the control of cell cycle [17–19]. The ψ_m was determined using JC-1 staining to determine the integrity of mitochondrial membrane. JC-1 aggregates (red fluorescence) prefer higher ψ_m in cells under normal condition. In response to the loss of ψ_m , the JC-1 monomers are dominant with green fluorescence. As a result, a profound increase of green fluorescence was induced after a 15-hour exposure to reevesioside F (Fig. 4A). The data demonstrated the loss of ψ_m and indicated the mitochondrial damage in leukemic cells responsive to reevesioside F.

Bcl-2 family members are key regulators of the integrity of mitochondrial membrane [19,20]. Bcl-2 and Mcl-1 are two central anti-apoptotic relatives, preventing apoptosis through antagonizing pro-apoptotic family members, including Bax and Bak. The BH3-only family proteins, including Bid, Bim and Noxa, are able to block anti-apoptotic members or directly stimulate Bax or Bak [20]. Reevesioside F resulted in the down-regulation of Mcl-1, up-regulation of Noxa and an increase of formation of truncated Bid (tBid) and tBim (Fig. 4B). Furthermore, reevesioside F also induced the formation of a 23-kDa cleaved Bcl-2 fragment, a product displaying pro-apoptotic activity [21] (Fig. 4B). The data correlated well with the loss of ψ_m , indicating that reevesioside F posed a stress and damage to the mitochondria.

3.4. Reevesioside F induces cytochrome c release into cytosol and causes association of several factors with mitochondria

The release of cytochrome *c* from the mitochondria to the cytosol is a critical process for the formation of apoptosome, a multimeric complex composed of Apaf-1, cytochrome *c*, ATP and pro-caspase-9 for the activation of mitochondrial apoptotic pathway [22,23]. Reevesioside F induced a dramatic down-regulation of mitochondrial Mcl-1 and an increase of cytosolic cytochrome *c*, which confirmed the mitochondrial stress and the subsequent apoptotic cell death (Fig. 5). Furthermore, recent studies have suggested that mitochondrial PARP plays a crucial role in the repair of mitochondrial DNA [24,25]. The detection of mitochondrial PARP revealed that reevesioside F induced the cleavage of mitochondrial PARP (Fig. 5). The effect might result in the damage of mitochondrial DNA and contribute to apoptosis.

3.5. Reevesioside F induces survivin down-regulation, α -tubulin acetylation and activation of caspase cascade

Caspase family of cysteine-aspartic proteases plays a central role in both initiation and execution of cell apoptosis. The data in Fig. 6 demonstrated that reevesioside F induced a profound increase of cleaved fragments of both initiator caspases (e.g., caspase-8 and -9) and executioner caspases (e.g., caspase-3 and -7). PARP, a substrate of both caspase-3 and -7, was also cleaved in response to reevesioside F (Fig. 7). Furthermore, the formation of activated H2A.X (γ -H2A.X) indicated the activation of a DNA damage response (Fig. 6). Altogether, the data suggest the caspase-dependent DNA damage in reevesioside F-mediated apoptotic cell death. Moreover, both intrinsic mitochondrial apoptotic pathway and extrinsic

death receptor apoptotic pathway were triggered, because the responsible caspases (e.g., caspase-9 and -8, respectively) were activated. However, reevesioside F had no effect on the protein expressions of death receptors (e.g., Fas, DR4 and DR5) and death ligands (e.g., FasL and TRAIL) (data not shown).

Survivin, the smallest and structurally unique member of inhibitor of apoptosis (IAP) family, serves as an endogenous inhibitor of caspase activation, thereby leading to negative regulation of apoptosis. The survivin protein is highly expressed in a wide variety of cancer cells, including leukemic cells [26]. The detection of survivin protein levels showed that reevesioside F induced a time-dependent decrease of survivin expression (Fig. 4B). It could contribute to the activation of caspase cascade. Notably, the overexpression of survivin significantly rescued reevesioside F-induced apoptosis ($56.8 \pm 5.1\%$ vs. $39.7 \pm 4.3\%$, $n = 3$, $P < 0.05$), confirming the crucial role of survivin. Furthermore, survivin has been suggested to regulate the stability of tubulin [27]. Western blotting showed that α -tubulin was significantly acetylated (Fig. 6) and translocated to the mitochondria (Fig. 5) after the exposure to reevesioside F. This activity may be responsible to apoptosis because there are studies providing evidence to support that tubulin association with mitochondria is part of apoptotic mechanism [28,29]. Histone deacetylase 6 (HDAC6) has emerged as a major deacetylase to cause α -tubulin deacetylation [30]. However, an HDAC activity assay demonstrated that reevesioside F did not display inhibitory activity against HDACs, including HDAC6 (data not shown).

3.6. Caspase-3 activity plays a major role in reevesioside F-mediated effect

Caspase-3 is the key executioner caspase in apoptotic cascade and, once activated, this enzyme cleaves a broad spectrum of substrates, committing the cell to apoptotic death [31]. To determine the functional role of caspase-3, the specific inhibitor z-DEVD-fmk was used. z-DEVD-fmk significantly blunted reevesioside F-induced apoptosis (Fig. 7A). Similar inhibitory effect also occurred to reevesioside F-mediated loss of Ψ_m (Fig. 7B). Furthermore, z-DEVD-fmk significantly inhibited the formation of cleaved Bcl-2 and tBid, and blocked the activation of caspases and PARP cleavage (Fig. 7C). Confocal immunofluorescence analysis demonstrated that z-DEVD-fmk profoundly inhibited reevesioside F-induced caspase-3 activation and γ -H2A.X nuclear localization (Fig. 7D). The data indicated that caspase-3 activity served as a central switch for the apoptotic signaling cascade.

4. Discussion

Cardiac glycosides are known to inhibit the Na^+/K^+ -ATPase, leading to an increase of intracellular Na^+ concentration which in turn triggers the exchange between intracellular Na^+ and extracellular Ca^{2+} , and ultimately results in an increase of calcium influx. The signaling pathway explains the increase of intracellular Ca^{2+} levels and contractility in cardiac muscle cells. However, reevesioside F did not increase the intracellular Ca^{2+} levels in leukemia cells. Furthermore, CGP-37157 (an inhibitor of $\text{Na}^+/\text{Ca}^{2+}$ exchanger) did not rescue reevesioside F-mediated effects. The data indicated that reevesioside F-induced anti-proliferative and apoptotic activities were not attributed to the inhibition of ion pumping activity by Na^+/K^+ -ATPase. Accumulating evidence suggests that certain subunits of Na^+/K^+ -ATPase

K⁺-ATPase may conduct non-pumping functions [3,5]. Both α_1 and β_1 subunits play a pump-independent role in epithelial junction formation and the regulation of tracheal tube-size [32]. β_1 subunit has been suggested to inhibit anchorage independent growth and block tumor formation in immunocompromised mice [33]. The present study showed that the α_3 subunit of Na⁺/K⁺-ATPase may play a central role on signaling the pump-independent anti-proliferative as well as apoptotic activities.

Mitochondria involve in a wide variety of cellular processes and functions, including energy production, lipid metabolism, storage of calcium ions, formation of hormones and synthesis of steroids. Therefore, mitochondria have been extensively documented to play a critical role in cell growth, survival, differentiation and apoptosis [17,18], and are considered as potential cellular targets in anticancer strategy to trigger cancer cell death [19]. Reevesioside F induced a dramatic loss of mitochondrial membrane potential and subsequent apoptosis identified by the detection of chromatin condensation and DNA fragmentation, indicating the apoptotic stress caused by mitochondrial damage. The mitochondrial apoptotic pathway is dependent on Bcl-2 family of pro-apoptotic and anti-apoptotic members that interact in a complex way to regulate the integrity of mitochondrial membrane [19,20]. Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Mcl-1, reside on outer mitochondrial membrane and prevent apoptosis through antagonizing pro-apoptotic family members, including Bax and Bak. The BH3-only family members (e.g., Bid, Bim and Noxa) can either block anti-apoptotic proteins or directly trigger Bax or Bak [20]. Reevesioside F induced the down-regulation of Mcl-1 protein and a profound up-regulation of Noxa, which led to the damage of outer mitochondrial membrane and triggered the release of cytochrome *c* to the cytoplasm, initiating the activation of caspase cascade and the execution of apoptosis [20,22].

It is noteworthy that reevesioside F induced the formation of truncated Bid (tBid). tBid contains the BH3 domain, serving as an active form of Bid and inducing the release of cytochrome *c* and apoptosis much more efficiently than that of Bid [34]. Notably, Mcl-1 can serve as a potent tBid-binding partner. It has been revealed that BH3 domain of tBid is necessary for Mcl-1 binding. In contrast, all three domains (BH1, BH2 and BH3) of Mcl-1 are essential for tBid binding [35]. However, reevesioside F caused a dramatic down-regulation of Mcl-1. This effect could allow more free tBid to undergo apoptotic program. The exposure to reevesioside F also induced the formation of truncated Bim (tBim). It has been reported that tBim is more potent than full length Bim protein in targeting Bcl-2 and inducing apoptosis [36,37]. Recent work has suggested that Bcl-2 activity can be regulated by its proteolytic cleavage fragment, a 23-kDa product with pro-apoptotic activity [21]. Reevesioside F resulted in the generation of 23-kDa Bcl-2 fragments as well. These data indicate that the truncated forms or active fragments of Bcl-2 family members may contribute to the amplification of apoptotic signaling cascade. Moreover, z-DEVD-fmk (a cell-permeable inhibitor of caspase-3), was able to blunt the formation of these active fragments. The data suggest that the executioner caspase-3 plays a central role in the positive feedback of apoptotic amplification in response to reevesioside F.

IAP family members bind to specific regions within caspase-3, -7 and -9 to block their activity. Survivin belongs to the IAP family. Some studies have reported the similar activity

of survivin to suppress caspase-3, -7 and -9 but not caspase-8 [38], while others have shown that survivin can inhibit the release of mitochondrial apoptotic factors, such as cytochrome *c* and apoptosis-inducing factor (AIF) [39]. Because of the role on inhibiting apoptosis, survivin has been suggested to be a potential target for cancer chemotherapy. Importantly, elevated protein levels of survivin have been detected in a lot of primary myeloid leukemia samples [38,40]. Moreover, survivin expression in acute myeloid leukemia has been documented to positively correlate with chemotherapy resistance [38,41]. These studies provide a rationale for the development of survivin-targeted therapies. In the present work, reevesioside F induced a profound down-regulation of survivin protein expression that happened prior to the activation of caspase cascade, suggesting that survivin served as an upstream regulator in the signaling pathway.

PARP is a predominantly nuclear enzyme that is involved in DNA repair in response to a wide variety of stresses and helps cells maintain their viability. PARP is one of the major cleavage targets for caspase-3 and the cleavage of PARP normally serves as an indication of cells undergoing apoptosis [42]. Recent work on investigating the role of PARP in mitochondria has demonstrated that the abrogation of mitochondrial localization of PARP leads to the accumulation of mitochondrial DNA damage. The study provides evidence suggesting that PARP binds to different regions throughout mitochondrial DNA and plays a role in mitochondrial DNA damage signaling and repair [24,25]. In this study, reevesioside F induced the cleavage of mitochondrial PARP. The effect may, at least partly, contribute to the apoptotic mechanism.

Mitochondrial membrane permeabilization is crucial for the release of pro-apoptotic mediators from mitochondria. The permeability transition pore is responsible for mitochondrial permeability. It has been suggested that mitochondrial tubulin may participate in the mechanism from cytoskeleton to mitochondria through voltage-dependent anion channel and act as a regulator of permeability transition pore. It is, therefore, suggested that mitochondrial tubulin contributes to cell apoptosis [28,29]. The data showed that reevesioside F induced an increase of α -tubulin translocation to mitochondria. The time-dependent effect was well correlated with apoptotic cell death. Furthermore, it has been revealed that, as compared with cellular tubulin, mitochondrial tubulin is enriched in acetylated α -tubulin [29]. Similarly, our data showed that reevesioside F induced an increase of mitochondria-associated acetylated α -tubulin, which was also in a good correlation with the undergoing of apoptosis. Taken together, the formation of acetylated α -tubulin and its mitochondrial association may be involved in reevesioside F-mediated apoptosis. However, whether these effects participate in the regulation of mitochondrial permeability transition pore warrants further investigation.

In conclusion, the data suggest that reevesioside F induces apoptosis in leukemia cells independent of intracellular Ca^{2+} mobilization. It results in the stress and damage to the mitochondria as well as the down-regulation of Mcl-1 and survivin proteins. The execution of apoptosis is achieved through a series of signaling pathways in which the activation of caspase-3 plays a critical role. The increased caspase-3 activity induces the formation of truncated Bcl-2 family members, which are more pro-apoptotic than the parent proteins, and

triggers further mitochondrial damage, leading to the amplification of caspase cascades and apoptosis in leukemia cells.

Acknowledgments

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Fig. 1.

Chemical structure of reevesioside F and identification of apoptotic effect. (A) Chemical structure of reevesioside F. (B) Graded concentrations of reevesioside F were added to the cells for 24 or 48 h. The cytotoxic effect was determined by MTT assay. (C) HL-60 cells were treated in the absence or presence of reevesioside F (100 nM) for the indicated times. After the treatment, the cells were fixed and stained with propidium iodide to analyze DNA content by FACSscan flow cytometer. (D) HL-60 cells were treated in the absence or presence of reevesioside F (100 nM) for the indicated times. DNA was electrophoresed on 2% agarose gel and stained with SYBRGreen. (E and F) HL-60 cells were treated with or without reevesioside F (100 nM) for 24 h. Microscopic examination was performed to detect apoptosis by nuclear staining with DAPI and Giemsa stain, respectively. The data are expressed as mean $\pm$ SEM of three to five independent experiments. ** $P < 0.01$ and *** $P < 0.001$ compared with the control.

Fig. 2.

Identification of the functional role of Na⁺/K⁺-ATPase α₃ subunit. (A) The expression of Na⁺/K⁺-ATPase α₃ subunit was detected using Western blot analysis. The anti-proliferative IC₅₀ values were determined using SRB assays except for HL-60 and Jurkat cells by MTT assays. (B and C) The parent and α₃ subunit knockdown HL-60 cells were treated with or without reevesioside F (100 nM) for 24 h. The cells were fixed and stained with propidium iodide to analyze DNA content by FACScan flow cytometer (B) or the cells were harvested and lysed for the detection of the protein expression by Western blot (C). The data are expressed as mean ± SEM of four independent experiments.

Fig. 3.

Effect of reevesioside F on intracellular Ca^{2+} concentration. HL-60 cells were incubated in the absence or presence of reevesioside F (100 nM) for 30 min (A), or treated with the indicated agents for 24 h (B and C). After the treatment, the cells were harvested for the detection of intracellular Ca^{2+} levels, ψ_m and apoptosis using flow cytometric analysis of the staining with fluo-3 AM, JC-1 and propidium iodide, respectively. The data are expressed as mean $\pm$ SEM of three independent experiments.

Fig. 4.

Effect of reevesioside F on ψ_m and related protein expression. HL-60 cells were incubated in the absence or presence of reevesioside F (100 nM) for the indicated times. Cells were incubated with JC-1 for the detection of ψ_m using FACSscan flow cytometric analysis (A), or cells were harvested and lysed for the detection of the indicated protein expression by Western blot (B). The expression was quantified using the computerized image analysis system ImageQuant (Amersham Biosciences). The data are expressed as mean $\pm$ SEM of three to four independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the control.

Fig. 5. Effect of reevesioside F on mitochondria-associated proteins. HL-60 cells were incubated in the absence or presence of reevesioside F (100 nM). The cells were harvested to separate mitochondrial and cytosolic fractions for Western blot analysis and subsequently, the expression was quantified using the computerized image analysis system ImageQuant (Amersham Biosciences). The data are expressed as mean $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the control.

Fig. 6.

Effect of reevesioside F on the expression of caspases and several proteins. HL-60 cells were incubated in the absence or presence of reevesioside F (100 nM) for the indicated times. The cells were harvested and lysed for the detection of the indicated protein expression by Western blot analysis. The expression was quantified using the computerized image analysis system ImageQuant (Amersham Biosciences). The data are expressed as mean $\pm$ SEM of three independent experiments. * P < 0.05 and *** P < 0.001 compared with the control.

Fig. 7.

Effect of z-DEVD-fmk on reevesioside F-induced apoptosis, loss of ψ_m and caspase expression. HL-60 cells were treated in the absence or presence of reevesioside F (100 nM) or z-DEVD-fmk (50 μ M) for 24 h. After the treatment, the cells were fixed and stained with propidium iodide to analyze DNA content (A), or the cells were incubated with JC-1 for the detection of ψ_m using flow cytometric analysis. The data are expressed as mean $\pm$ SEM of three independent experiments (B). After the treatment of the indicated agent for 24 h, the cells were harvested and lysed for the detection of the indicated protein expression by Western blot analysis. The data are representative of three independent experiments (C). Double immunofluorescent staining for caspase 3 (green) and γ -H2A.X (red) was performed

using fluorescence microscopic examination. DAPI staining was applied for identification of nuclei (D). *Bar*, 20 μm .