Bufotalin sensitizes death receptor-induced apoptosis via Bid- and STAT1-dependent pathways

PORNTHIP WAIWUT, AKIKO INUJIMA, HIROKI INOUE, IKUO SAIKI and HIROAKI SAKURAI

Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan

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Abstract. Tumor necrosis factor-alpha (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL) are apoptosis-inducing ligands that stimulate death receptors. In this study, we investigated the effects of bufotalin, a major compound in toad venom, on sensitizing TNF-α and TRAIL-induced apoptosis of HeLa cells. Bufotalin promoted death receptor-mediated cell death, especially TRAIL-induced apoptosis, through activation of caspase-3 and PARP-1. Mitochondrial Bid-dependent pathway was activated in TNF-α-induced cell death. Cotreatment of bufotalin with TRAIL resulted in the downregulation of anti-apoptotic proteins, including Bcl-XL, Mcl-1, survivin and XIAP, and the up-regulation of MAPKs and TRAIL receptor DR5. In addition, phosphorylation of STAT1 was strongly inhibited by bufotalin. Moreover, DR5 expression was induced by knocking down the STAT1 expression. Moreover, the TRAIL-induced apoptotic response was promoted by STAT1 siRNA. Our results demonstrated that bufotalin is a powerful sensitizer of death receptor-induced apoptosis in cancer cells.

Introduction

Many chemotherapeutic agents induce tumor cell death by apoptosis. Apoptosis can be initiated by two distinct pathways: through mitochondria (intrinsic pathway) or through death receptors on the cell surface (extrinsic pathway). Induction of apoptosis via the intrinsic pathway leads to the breakdown of mitochondrial membrane potential and release of cytochrome c, and subsequently results in the activation of procaspase 9 within the so-called apoptosome (1). In addition, death ligands binding to the death receptors, including Fas, tumor necrosis factor receptor 1 (TNF-R1) or TNF-related apoptosis-inducing ligand (TRAIL) receptor DR4/5, trigger extrinsic apoptosis pathways.

TNF-α binds to TNF-R1, which recruits the adaptor molecule TRADD and FADD to activate a caspase cascade from the upstream initiator caspase-8 to the downstream effector caspase-3 (2,3). TRAIL is known to induce apoptosis in a variety of tumor cells through its action with two distinct receptors, DR4 and DR5. Bid, a member of the Bcl-2 family, is the main molecular linker connecting the death receptor pathway and the mitochondrial pathway. Bid can activate mitochondria via direct interaction with Bax or Bak. Cytosolic Bid is cleaved by caspase-8 at the amino terminus to generate a truncated form of Bid (tBid) that mediates cytochrome c release from mitochondria, which serves as an amplification signal by activating downstream effector caspases, including caspase-3 (4-8). Pro-caspase-3 can also be directly cleaved by caspase-8 in a mitochondrion-independent manner. In turn, effector caspases are responsible for cleaving cellular proteins, such as poly(ADP-ribose) polymerase (PARP) (9). Caspase cascade can be blocked by anti-apoptosis proteins, such as cIAP1/2, Bcl-xl, Mcl-1, Survivin and XIAP (10).

Chan Su, toad venom, is a traditional Chinese medicine from the skin secretions of Bufo bufogargarizans Cantor and B. melsanostictus Schneider. It has been used to treat various diseases such as cardiac illness, pain, and cancer in China and other Asian countries (11,12). Bufadienolides, the major active constituents of Chan Su, are C-24 steroids, the characteristic structural feature of which is a doubly unsaturated six-membered lactone ring (β-pyrone) at position 17β. Furthermore, these compounds are characterized by the trans-junction of rings B and C and usually the cis-junction of rings C and D. More than 40 bufadienolides have been identified, including bufalin, bufotalin, gamabufotalin, cinobufutoxin and resinobufogenin (13,14). Bufotalin (Fig. 1A) has been reported to induce apoptosis in human hepatocellular carcinoma (HCC) Hep 3B cells (15); however, its molecular mechanisms in the death receptor signaling pathway remain unknown.

The present study investigated the effect of bufotalin on TNF-α- and TRAIL-induced apoptosis in HeLa cells. We found that activation of the caspase-induced apoptosis pathway by bufotalin was dependent on Bid and STAT1 pathways.

Correspondence to: Dr Hiroaki Sakurai, Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
E-mail: hsakurai@inm.u-toyama.ac.jp

Abbreviations: PARP-1, poly(ADP-ribose) polymerase 1; Bcl-XL, B-cell lymphoma-extra large; Mcl-1, myeloid cell leukemia sequence 1; XIAP, X-linked inhibitor of apoptosis protein; MAPKs, mitogen-activated protein kinases; DR5, death receptor 5; STAT1, signal transducers and activators of transcription-1; siRNA, small interfering RNAs

Key words: TNF-α, TRAIL, Bid, STAT1
Materials and methods

Antibodies and reagents. The anti-phospho-specific antibodies against p65 (Ser-536), ERK (Thr-202, Tyr-204), p38 (Thr-180/Tyr-182), JNK (Thr-183/Tyr-185), STAT1 (Tyr-701), STAT3 (Tyr-705), and total antibodies against PARP-1, Mcl-1, Bid, XIAP Bcl-XL Survivin, STAT3 and caspase-3, -7, -8, -9 were purchased from Cell Signaling Technologies. Antibodies against p38 (C-20-G), JNK, ERK1 (C-16), p65 (C-20-G), STAT1, and actin (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TNF-α was obtained from R&D Systems. Recombinant human TRAIL was purchased from Peprotech (London, UK). Bufotalin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture and cytotoxicity assay. HeLa cells were maintained in Dulbecco’s modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. Cell viability was quantified using the cell proliferation reagent WST-1 assay. Cells were plated in 96-well microplates at 6x10³ cells/well, and then incubated for 24 h. Bufotalin-containing medium was added to the wells, and cells were incubated for 30 min, and then stimulated with TNF-α. After 24-h incubation, 10 µl of WST-1 solution was added, and absorbance was measured at 450 nm.

Small interfering RNAs and transfection. The siRNA for human STAT1 and non-targeting siRNA were purchased from Invitrogen Life Technologies (Carlsbad, CA). HeLa cells were transfected with siRNAs at a final concentration of 20 nM using Lipofectamine reagents (Invitrogen). At 72 h after transfection, cells were stimulated.

Preparation of cell extracts. Cells were treated with bufotalin and TNF-α, and whole cell lysates were prepared with lysis buffer (25 mM HEPES pH 7.7, 0.3 mM MgCl₂, 0.2 mM EDTA, 10% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Cell lysate was collected from the supernatant after centrifugation at 14,000 rpm for 10 min. Nuclear extracts were prepared as described previously (12). In brief, cells were suspended in 420 µl buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin] and chilled on ice for 15 min. Next, 25 µl of 10% Nonidet P-40 was added and

Figure 1. Effects of bufotalin on TRAIL- and TNF-α-induced cell death. (A) Chemical structure of bufotalin. (B) HeLa cells were treated with bufotalin at 0.01 and 0.1 µM in the presence or absence of TNF-α (20 ng/ml) or TRAIL (200 ng/ml) for 24 h. Cell viability was determined by WST-1 assay. *p<0.001. (C) Cell morphology was examined in BZ8000 micrographs.
the suspension was vigorously vortexed for 10 sec. The nuclear pellets were suspended in 50 µl buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin). The mixture was kept on ice for 15 min with frequent agitation. Nuclear extracts were prepared by centrifugation at 15,000 rpm for 5 min.

Preparation of mitochondria and cytosol proteins. The cytosolic and mitochondrial protein fractions were isolated according to a previously reported procedure (24). Briefly, after treatment as indicated, cells were scraped in ice-cold homogenization buffer. The cells were then resuspended in 5 volumes of ice-cold extract buffer A (20 mM HEPES, 20 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM Na3VO3, pH 7.5, and 1X protease inhibitor cocktail) and homogenized. The homogenates were centrifuged at 750 g for 10 min, and then the supernatant was collected and centrifuged at 10,000 g for 15 min to obtain mitochonidrion pellets. The supernatants were further centrifuged at 100,000 g for 1 h to collect the supernatants (cytosolic fraction).

Immunoblotting. Cell lysate was resolved by SDS-PAGE and transferred to an Immobilon-P-nylon membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) and probed with primary antibodies. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (Dako, Glostrup, Denmark), and visualized with the enhanced chemiluminescence system (Amersham Biosciences).

Immunoprecipitation. Cell lysates were diluted with an equal volume of dilution buffer [20 mM HEPES (pH 7.7), 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin]. After centrifugation, lysates were incubated with anti-DR4 and DR5 antibody (Santa Cruz Biotechnology) on ice for 1.5 h and then rotated with protein G-Sepharose (Amersham Biosciences) at 4˚C for 2 h. The Sepharose beads were washed twice with washing buffer (a 1:1 mixture of whole-cell lysate buffer and dilution buffer).

Real-time RT-PCR. Total RNA was prepared using the RNeasy Mini kit (Qiagen). First-strand cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen). The cDNA was amplified quantitatively using SYBR Premix Ex Taq (Takara-Bio, Otsu, Japan), using the following primers: DR5 sense: 5’-GCACCACGACCAGAAA-3’; antisense: 5’-CACCGACCTTGCACCAT-3’. Real-time quantitative RT-PCR was performed using an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA). All data were normalized to GAPDH mRNA.

Results

Bufotalin sensitizes HeLa cells to TRAIL and TNF-α-induced cell death. We first examined the effects of bufotalin on TNF-α and TRAIL-induced cytotoxicity (Fig. 1B). HeLa cells were resistant to TNF-α- and TRAIL-induced cell death. Bufotalin at 0.1 µM alone showed weak cytotoxicity in a WST-1 assay; however, combination with TNF-α or TRAIL markedly increased the sensitivity to these cytokines. Phase-contrast microscopy supported the results of a proliferation assay (Fig. 1C). The results indicate that bufotalin markedly enhanced both TNF-α and TRAIL-induced cancer cell deaths.

Bufotalin enhances TNF-α-induced apoptosis through Bid-dependent pathway. We investigated the effect of bufotalin on TNF-α-induced apoptosis by determining the cleavage of caspase-3 and PARP-1. Cells were pretreated with bufotalin at 0.1 µM for 30 min, and then stimulated with TRAIL (200 ng/ml) for 4 h. Western blotting performed by using anti-caspase-8, Bid, BCL-2, cytochrome c, and actin antibodies. (C) Effects of bufotalin on TNF-α-induced NF-κB and MAPK signaling pathways were examined using anti-phospho-p65, Erk, p38, JNK and actin antibodies.

Figure 2. Effects of bufotalin on TNF-α-induced apoptosis. HeLa cells were pretreated with bufotalin at various concentrations (0.01, 0.03 and 0.1 µM) for 30 min, and then stimulated with TNF-α (20 ng/ml) for 4 h. Whole cell extract was prepared, fractionated and analyzed by Western blotting. (A) Western blot results using anti-caspase-3, PARP and actin antibodies. Arrows indicate cleaved forms of caspases-3 and PARP. (B) Effects of bufotalin on TNF-α-mediated Bid-dependent pathway. Cells were pretreated with bufotalin at 0.1 µM for 30 min, and then stimulated with TRAIL (200 ng/ml) for 4 h. Western blotting performed by using anti-caspase-8, Bid, BCL-2, cytochrome c, and actin antibodies. (C) Effects of bufotalin on TNF-α-induced NF-κB and MAPK signaling pathways were examined using anti-phospho-p65, Erk, p38, JNK and actin antibodies.
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(Fig. 2B). In addition, mitochondrial-type Bid and subsequent cytochrome c release were increased. In contrast, activation of NF-κB p65 and three MAPK cascades (p38, Erk and JNK) was not affected (Fig. 2C). This result suggested that bufotalin potentiated TNF-α-induced apoptosis through a Bid-dependent pathway.

Bufotalin sensitizes TRAIL-induced apoptosis. Similar to the effect on TNF-α response, bufotalin promoted TRAIL-induced cleavage of caspase-8, -3, -7 and PARP-1 in a concentration-dependent manner (Fig. 3). In addition, the expressions of anti-apoptotic proteins Bcl-XL, Mcl-1, Survivin and XIAP were reduced markedly by treatment with both TRAIL and bufotalin. Moreover, total Bid, a mitochondrion-dependent apoptotic protein, was also decreased. Because MAPKs and NF-κB play important roles in modulating apoptosis, we determined if such signaling pathways are involved in bufotalin-induced cell death. Consequently, although treatment with TRAIL or bufotalin alone had no effect on the phosphorylated forms of p38, ERK, and JNK, the combination of TRAIL and bufotalin resulted in synergistic activation of p38, ERK, and JNK at 4 h (Fig. 3). These results indicated that bufotalin synergized the decrease in anti-apoptotic protein expression in the presence of TRAIL.

Effect of bufotalin on the expression of TRAIL receptor DR5. In order to ascertain the mechanism by which bufotalin sensitizes HeLa cells to TRAIL, we next investigated whether the compound enhances the expression of death receptors; therefore, cells treated with bufotalin and TRAIL were analyzed by RT-PCR to quantify DR5 mRNA expression. As shown in Fig. 4A, 4-h incubation with bufotalin alone slightly enhanced DR5 mRNA expression and TRAIL enhanced this activity. Moreover, treatment with TRAIL resulted in synergistic activation of p38, ERK, and JNK at 4 h (Fig. 3). These results indicated that bufotalin synergized the decrease in anti-apoptotic protein expression in the presence of TRAIL.

Bufotalin inhibits TRAIL-induced STAT1 phosphorylation. It has been reported that JAK and STAT pathways could suppress apoptosis by inhibiting DR5 expression. In the present study we
investigated the effects of bufotalin on TRAIL-mediated phosphorylation of STAT1 and STAT3. Fig. 5A shows that cotreatment with bufotalin and TRAIL suppressed the phosphorylation of both STAT1 and STAT3 in a concentration-dependent manner. Since TRAIL signaling is often accompanied by the expression of DR5, we next investigated the functional significance of STAT1 in sensitization to TRAIL. DR5 mRNA expression was strongly induced by TRAIL in cell down-regulating STAT1 expression (Fig. 5B). In addition, TRAIL-induced caspase-3 and PARP activation was enhanced by STAT1 knock-down, indicating that down-regulation of STAT1 is sufficient to sensitize cells to TRAIL-induced apoptosis.

Discussion

Bufotalin, one of the bufadienolides isolated from Formosan Chan Su, has been used as an important traditional Chinese medicine for heart failure and pain. Bufadienolides are the major bioactive constituents of Chan Su, exhibited strong anticancer, cardiotonic and anesthetic activities (12). In particular, they have significant cytotoxic activities against various human cancer cells, such as gastric and liver cancer cells (16,17). A previous study reported that bufotalin induced apoptosis of Hep 3B cells (15); however, the mechanism of bufotalin for death-receptor-induced apoptosis is not clearly understood. Consequently, this study investigated the effects and molecular mechanisms of bufotalin in sensitizing TNF-α and TRAIL-mediated apoptosis of HeLa cells.

Death receptor-mediated tumor cell death, either alone or in combination with other anticancer drugs, is considered a new approach for anticancer therapy. TNF-α and TRAIL are death ligands playing an important role in apoptosis induction through their death receptor pathways (3). In the present study, we found that bufotalin markedly enhanced TRAIL and TNF-α-induced cell death through caspase-3 and PARP-1 activation.

In the death receptor-mediated apoptosis signaling pathway, NF-κB is widely known for its ubiquitous roles in cell survival and apoptosis. NF-κB protects cells from apoptosis by transcriptional activation of survival factors, such as anti-apoptotic proteins (18). Our results showed that bufotalin had no effect on TNF-α- and TRAIL-induced NF-κB activation (data not shown). In other pathways, the contribution of MAPKs has been extensively documented. As indicated previously, p38 ERK and JNK activation leads to the mitochondrial apoptotic pathway (19,20), and JNK activation promotes the processing of Bid to a proapoptotic cleaved product (21). Previous experiments have reported that JNK ERK and p38 activation contributed to the sensitization of TRAIL-induced apoptosis (22-24). Our results indicated that co-treatment with bufotalin and TRAIL resulted in enhanced apoptosis through delayed activation of...
MAPKs, whereas there was no effect of bufotalin on TNF-α-induced rapid activation of MAPKs. As bufotalin has no effect on TNF-α-induced NF-xB and MAPKs, we therefore suggest that the signaling pathway may be involved in the mitochondrial apoptotic pathway. In our study, bufotalin induced TNF-induced cleavage of caspase-3 and PARP-1 through Bid activation, indicating the participation of mitochondrial dependent apoptotic machinery in cell death.

Previous it was reported that a decrease in the phosphorylated form of STAT3 could be responsible for the enhanced expression of death receptor (25). In this study, we found that bufotalin together with TRAIL inhibited STAT1 and STAT3 phosphorylation, and induced DR5 mRNA and protein expression, indicating that bufotalin may enhance TRAIL-induced death receptor expression through STAT3. Moreover, we found that downregulation of STAT1 with siRNA caused upregulation of DR5 mRNA and the protein level, which was sufficient to sensitize cells to TRAIL-induced apoptosis. This finding indicated that bufotalin sensitizes TRAIL-induced apoptosis via STAT1-mediated DR5 upregulation. To further study the mechanism of bufotalin-induced apoptosis, both anti-apoptotic molecules (Bcl-XL, Mcl-1, Survivin and XIAP) and the pro-apoptotic molecule Bid were examined in this study. Among them, we demonstrated that co-treatment with bufotalin and TRAIL was able to downregulate Bcl-XL, McIL-1, Survivin XIAP and Bid.

In conclusion, bufotalin can sensitize both TNF-α- and TRAIL-induced apoptosis in HeLa cells. Bufotalin participated in TNF-α-induced apoptosis via a Bid-dependent pathway by activating caspase-8, Bid, cytochrome c and caspase-3, respectively, whereas bufotalin sensitized TRAIL-induced apoptosis through MAPK activation and STAT1-mediated upregulation of DR5.

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