Berberine sensitizes TRAIL-induced apoptosis through proteasome-mediated downregulation of c-FLIP and Mcl-1 proteins

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Abstract. Berberine (BBR) is an isoquinoline alkaloid which has a wide spectrum of clinical applications including anti-tumor, anti-microbial and anti-inflammatory activities. In this study, we showed that co-treatment with subtoxic doses of BBR and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis in human renal cancer cells, Caki cells, but not in normal tubular kidney cells. Treatment of Caki cells with BBR resulted in downregulation of c-FLIP and Mcl-1 proteins in a dose-dependent manner. The BBR-induced downregulation of c-FLIP and Mcl-1 proteins were involved in proteasome dependent pathways, which was confirmed by the result that pre-treatment with the proteasome inhibitor MG132 inhibited berberine-induced downregulation of the c-FLIP and Mcl-1 proteins. Pretreatment with N-acetyl-l-cysteine (NAC) significantly inhibited the cell death induced by the combined treatment with BBR and TRAIL as well as recovered the expression levels of c-FLIP and Mcl-1 downregulated by treatment with BBR. These results suggested that BBR-stimulated TRAIL-induced apoptosis is dependent on the generation of reactive oxygen species through the downregulation of c-FLIP and Mcl-1 proteins. In conclusion, this study demonstrates that BBR enhances TRAIL-mediated apoptosis in human renal cancer cells by ROS-mediated c-FLIP and Mcl-1 down-regulation.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a potential new anticancer drug. TRAIL induces apoptosis in various types of cancer cells in vitro and in vivo, but has little or no toxicity against normal cells, which is supported by the presence of large numbers of decoy receptors on normal cells (1,2). A soluble recombinant TRAIL is undergoing a phase I clinical trial for the treatment of solid tumors (3). However, recent studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL (4-6), indicating that the single treatment with TRAIL may be insufficient for cancer therapy. Some studies demonstrated that several dietary compounds including resveratrol (6), withaferin A (7), and curcumin (8) sensitize different cancer cells to TRAIL-mediated apoptosis.

Berberine (BBR), a natural alkaloid, is isolated from many medicinal herbs, such as the Chinese herb Huanglian, berberis aquifolium, and berberis vulgaris (9). It has been shown to exhibit multiple pharmacological activities such as inhibition of DNA synthesis, G1 cell cycle arrest, and anti-cancer effect (10,11). BBR has been reported to inhibit expression of cyclooxygenase-2 (COX-2), to exert anti-metastatic properties in non-small lung cancer cells, as well as to induce apoptosis via promoting the expression of caspase, apoptosis-inducing factor (AIF) and endonuclease G in human tongue squamous carcinoma cancer cells (12). In addition, BBR also sensitizes human glioma cells to ionizing radiation in vitro (13). It was also reported that co-administration with BBR and doxorubicin in xenografted human tongue squamous carcinoma cancer reduced tumor growth at the concentration of doxorubicin without any loss of body weight (14).

Although a large number of studies also showed that BBR possesses anti-tumor activity, the anti-cancer effect of the combination of BBR and TRAIL is not clear. In the present study, we examined the therapeutic potential of BBR on TRAIL-mediated apoptosis and the potential application of BBR as a complimentary therapeutic agent for human renal cancer cells.

Materials and methods

Cells and materials. Caki cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).
Primary culture of human mesangial cells (Cryo NHMC) and its corresponding growth medium (CC-3146 MsGM) were purchased from Clonetics (San Diego, CA). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml gentamicin. BBR was directly added to cell cultures at the indicated concentrations. Anti-Bcl-2, anti-PARP, anti-procaspase-3, anti-DR5, anti-Mcl-1, and anti-actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-c-FLIP antibody was purchased from Alexis Corporation (San Diego, CA). Berberine was obtained from Sigma Chemical Co. Recombinant human TRAIL/Apo2 ligand (the nontagged 19 kDa protein, amino acid 114-281) was purchased from KOMA Biotech Inc. (Seoul, Korea).

Western blotting. Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl₂, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Cell count and flow cytometry analysis. Cell counts were performed using a hemocytometer. Approximately 1×10^6 Caki cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by flow cytometry. The accumulation of sub-G1 phase cells, compared with Caki cells treated with BBR and TRAIL, resulted in a markedly increased accumulation of sub-G1 phase cells, compared with Caki cells treated with BBR or TRAIL alone (Fig. 1A). In addition, the combined treatment of Caki cells with BBR and TRAIL strongly led to a reduction of the protein levels of 32-kDa precursor together with a concomitant cleavage of PARP, a substrate protein of caspases (Fig. 1B). Next, we analyzed nuclear condensation, which is another hallmark of apoptosis. Combinational treatment with BBR plus TRAIL induced the nuclear condensation in Caki cells. In contrast, nuclear condensation in Caki cells treated with TRAIL alone or BBR alone was barely detected (Fig. 1C). To examine whether co-treatment with BBR and TRAIL affected renal cancer ACHN cells, we investigated the effect of the combined treatment with BBR and TRAIL in human renal cancer cell line, Caki cell. Co-treatment of Caki cells with BBR and TRAIL resulted in a markedly increased accumulation of sub-G1 phase cells, compared with Caki cells treated with BBR or TRAIL alone (Fig. 1A). In addition, the combined treatment of Caki cells with BBR and TRAIL strongly led to a reduction of the protein levels of 32-kDa precursor together with a concomitant cleavage of PARP, a substrate protein of caspases (Fig. 1B). Next, we analyzed nuclear condensation, which is another hallmark of apoptosis. Combinational treatment with BBR plus TRAIL induced the nuclear condensation in Caki cells. In contrast, nuclear condensation in Caki cells treated with TRAIL alone or BBR alone was barely detected (Fig. 1C). To examine whether co-treatment with BBR and TRAIL affected renal cancer ACHN cells, we investigated the effect of the combined treatment with BBR and TRAIL in ACHN cell. Co-treatment of ACHN cells with BBR and TRAIL resulted in a markedly increased accumulation of sub-G1 phase cells, compared with ACHN cells treated with BBR or TRAIL alone (Fig. 1D). Next, we investigated whether co-treatment with BBR and TRAIL affected TCMK-1 normal renal tubular epithelial cell. Interestingly, TCMK-1 cells were resistant to 60 μM BBR or 100 ng/ml TRAIL alone, and cell morphologies were not significantly affected by treatment with a combination of 60 μM BBR and 100 ng/ml TRAIL (Fig. 1E).
Figure 1. BBR sensitizes renal cancer cells to TRAIL-mediated apoptosis. (A) Caki cells were treated for 24 h with TRAIL (100 ng/ml) in either the absence or the presence of BBR (60 μM). After 24 h of treatment, apoptosis was analyzed as a sub-G1 fraction by FACS. *p<0.05 for BBR+TRAIL-treated cells versus control, BBR-, or TRAIL-treated cells by ANOVA. (B) Activation of caspases and cleavage of PARP in BBR-sensitized TRAIL-induced apoptosis. Cells were treated with the indicated concentrations of BBR and TRAIL. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot analysis for PARP, pro-caspase-3, and actin for normalization. (C) After treatment with BBR plus and TRAIL for 24 h, cells were harvested and washed with PBS and exposed to 300 nM 4',6-diamidino-2-phenylindole (DAPI) at room temperature in the dark for 15 min. Samples were observed under a fluorescence microscope. The healthy cell had an oval-shaped cell body, and its chromatin stained dimly and occupied the majority of the cell body. The chromatin of an apoptotic cell was condensed, intensely stained, or shifted to the periphery of the cell body. The morphologies of cells were determined by interference light microscopy (magnification, x200). (D) ACHN cells were treated for 24 h with TRAIL (100 ng/ml) in either the absence or the presence of BBR (60 μM). After 24 h of treatment, apoptosis was analyzed as a sub-G1 fraction by FACS. *p<0.05 for BBR+TRAIL-treated cells versus control, BBR-, or TRAIL-treated cells by ANOVA. (E) Mouse tubular normal TCMK-1 cells were treated for 24 h with TRAIL (100 ng/ml) in either the absence or the presence of BBR (60 μM). After 24 h of treatment, apoptosis was analyzed as a sub-G1 fraction by FACS.

Figure 2. The combined treatment with BBR plus TRAIL induced apoptosis is mediated by caspase-dependent pathway. (A) Effect of z-VAD-fmk on apoptosis induced by BBR plus TRAIL. Caki cells were incubated with 50 μM z-VAD-fmk or solvent for 1 h before treatment with BBR (60 μM) and/or TRAIL (100 ng/ml) for 24 h. DNA contents of treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. Data are mean values obtained from three independent experiments and bars represent standard deviation. *p<0.05 compared to BBR plus TRAIL-treated cells. (B) Effect of z-VAD-fmk on caspase activation in BBR plus TRAIL treated cells. Cells were treated with the indicated concentrations of BBR and TRAIL. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot analysis for procaspase-3 and PARP. The proteolytic cleavage of PARP is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results.
Combinational treatment with BBR plus TRAIL-induced apoptosis was mediated by caspase-dependent pathway. We examined whether the activation of caspase pathway plays a critical role in BBR plus TRAIL-induced apoptosis. As shown in Fig. 2A, BBR plus TRAIL-induced apoptosis was completely prevented by pretreatment with a general and potent inhibitor of caspases, z-VAD-fmk, as determined by FACS analysis. These results suggest that the combined treatment with BBR and TRAIL-induced apoptosis was mediated by caspase-dependent apoptosis pathways. We also found that z-VAD-fmk prevented these caspase-related events such as downregulation of procaspase-3 and cleavage of PARP (Fig. 2B).

BBR downregulates Mcl-1 and c-FLIP protein levels at post-translational level. To investigate the underlying mechanisms involved in the combined treatment with BBR and TRAIL-induced apoptosis, we analyzed the expression levels of various apoptosis regulating proteins. While protein levels of XIAP, c-FLIP, and Mcl-1 were remarkably reduced in response to BBR plus TRAIL, the levels of death receptor 5 (DR5), c-IAP1, Bcl-2, and Bcl-xL, did not alter (Fig. 3A). Next, we examined whether the decreased expressions of XIAP, c-FLIP and Mcl-1 proteins were related to caspase activations. Pretreatment with z-VAD-fmk completely recovered XIAP protein to basal level. In contrast, z-VAD-fmk partly blocked BBR plus TRAIL-induced downregulations of

Figure 3. BBR downregulates Mcl-1 and c-FLIP protein levels at post-translational levels. (A) Caki cells were treated with the indicated concentrations of BBR for 24 h and harvested in lysis buffer. The equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE. Western blotting was performed using anti-DR5, anti-XIAP, anti-Mcl-1, anti-Bcl-2, anti-c-IAP1, anti-c-FLIPs, anti-c-FLIP_L, Bcl-xL antibodies, or anti-actin antibody to serve as control for the loading of protein level. (B) Effect of z-VAD-fmk on protein expression induced by BBR plus TRAIL. Caki cells were incubated with 50 μM z-VAD-fmk or solvent for 1 h before treatment with BBR (60 μM) and/or TRAIL (100 ng/ml) for 24 h. The cells were harvested in lysis buffer, and then equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE. Western blotting was performed using anti-XIAP and anti-c-FLIP, anti-Mcl-1 antibodies, or anti-actin antibody to serve as control for the loading of protein level. (C) Caki cells were treated with the indicated times for 24 h and harvested in lysis buffer and equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE. Western blotting was performed using anti-c-FLIP, anti-c-FLIP_L, anti-Mcl-1, anti-Bcl-2 and anti-Bcl-xL antibodies or anti-actin antibody to serve as control for the loading of protein level. (D) Caki cells were treated with the indicated concentrations of BBR for 24 h. Total RNA was isolated and RT-PCR analysis was performed as described in Materials and methods. A representative study is shown; two additional experiments yielded similar results.
the degradations of c-FLIP and Mcl-1 proteins were facilitated by BBR treatment (Fig. 4A), implying that BBR treatment caused reduction of the c-FLIP and Mcl-1 protein stability. Based on this result, we hypothesized that c-FLIP and Mcl-1 proteins would be degraded by the proteasome-dependent pathway. To confirm this hypothesis, we treated the cells with the proteasome inhibitor MG132. Caki cells were treated with BBR (60 μM) plus CHX in the absence or presence of MG132 (0.5 μM). As expected, the decreased protein levels of c-FLIP and Mcl-1 in BBR-treated Caki cells were almost recovered to basal levels by MG132 treatment (Fig. 4B).

**Figure 4. Decreased expressions of c-FLIP and Mcl-1 proteins are caused by the activation of proteasome-dependent pathway in BBR-treated cell.** (A) Caki cells were treated with 60 μM BBR in the presence or absence of CHX for the indicated times. Western blotting was performed using anti-c-FLIP and anti-Mcl-1 and anti-actin antibody to serve as control for the loading of protein level. (B) Caki cells were treated with 60 μM BBR plus CHX in the presence or absence of 0.5 μM MG132 for 24 h. Western blotting was performed using anti-c-FLIP, and anti-Mcl-1 antibodies or anti-actin antibody to serve as control for the loading of protein level.

c-FLIP and Mcl-1 proteins, indicating that the decreased c-FLIP and Mcl-1 protein levels were partly caused by caspase activation (Fig. 3B). These results suggested the possibility that the decrease of c-FLIP and Mcl-1 proteins were partly mediated by caspase-independent pathways. To ascertain the downregulation of c-FLIP and Mcl-1 by BBR in Caki cells, we carried out time kinetics studies. As shown in Fig. 3C, protein levels of c-FLIP and Mcl-1 were markedly decreased by the indicated concentrations of BBR-treated Caki cells in a dose-dependent manner. To further elucidate the mechanism responsible for the changes in amounts of c-FLIP and Mcl-1 proteins, we determined the levels of c-FLIP and Mcl-1 mRNA by RT-PCR. c-FLIP and Mcl-1 mRNA levels remain constant throughout the BBR treatment at different doses in Caki cells (Fig. 3D). These results suggest the possibility that BBR-mediated degradation of total c-FLIP and Mcl-1 proteins might be regulated by the post-transcriptional levels.

**Decreased expressions of c-FLIP and Mcl-1 proteins were caused by the activation of proteasome-dependent pathway in BBR-treated cells.** To further clarify the underlying mechanisms of the decreased c-FLIP and Mcl-1 protein level in BBR-treated cells, we analyzed the stability of c-FLIP and Mcl-1 proteins. Caki cells were treated with cycloheximide (CHX) plus BBR for different periods of time. We found that the degradations of c-FLIP and Mcl-1 proteins were facilitated by BBR treatment (Fig. 4A), implying that BBR treatment caused reduction of the c-FLIP and Mcl-1 protein stability. Based on this result, we hypothesized that c-FLIP and Mcl-1 proteins would be degraded by the proteasome-dependent pathway. To confirm this hypothesis, we treated the cells with the proteasome inhibitor MG132. Caki cells were treated with BBR (60 μM) plus CHX in the absence or presence of MG132 (0.5 μM). As expected, the decreased protein levels of c-FLIP and Mcl-1 in BBR-treated Caki cells were almost recovered to basal levels by MG132 treatment (Fig. 4B).

**BBR-stimulated TRAIL-induced apoptosis appears to be dependent on the formation of reactive oxygen species (ROS) via the downregulation of c-FLIP and Mcl-1.** Numerous investigations have documented that ROS may play an important role during apoptosis induction (15,16). It has been reported that BBR increases ROS production in various cancer cells (10,17,18). Therefore, we examined whether ROS generation might be involved in BBR plus TRAIL-induced apoptosis. H2DCFDA-based fluorescence microscope detection showed that treatment of Caki cells with BBR increased intracellular ROS levels, which were prevented by anti-oxidant N-acetyl-L-cysteine (NAC) pretreatment (Fig. 5A). We next investigated whether ROS generation is directly associated with BBR plus TRAIL-induced apoptosis. As shown in Fig. 5B, pretreatment with NAC markedly blocked BBR plus TRAIL-induced apoptosis. However, NAC pretreatment recovered the decreased expression levels of c-FLIP by BBR treatment to basal levels, BBR-induced downregulation of Mcl-1 protein was partly blocked by NAC treatment (Fig. 5C). These results suggest the possibility that BBR-stimulated downregulation of c-FLIP protein appears to be dependent on the generation of reactive oxygen species for downregulation of c-FLIP through proteasomal activation.

**Discussion**

In the present study, we demonstrate for the first time that treatment of renal cancer cells with BBR in combination with TRAIL synergistically induced apoptosis. c-FLIP and Mcl-1 were downregulated when BBR and TRAIL were administered together, which was caused by facilitating degradation of c-FLIP and Mcl-1 proteins. In addition, we also found that treatment with BBR in combination with TRAIL of Caki cells led to ROS generation, as shown using the fluorescent dyes, H2DCFDA, which production of ROS by BBR treatment seems to be critical for c-FLIP and Mcl-1 downregulation. Furthermore, combinatory treatment with BBR plus TRAIL synergistically enhanced apoptosis in renal cancer ACHN cells without any cytotoxicity in normal tubular kidney cells.

Several studies demonstrate that TRAIL almost selectively induces apoptosis in cancer cells with no or minimal toxicity on non-neoplastic cells (1,5). However, treatment with TRAIL alone may be insufficient for cancer therapy because some cancers display resistance to TRAIL. In addition, regular consumption of natural compounds such as tea, herbs, gingers, and spices is associated with reduced risk of cancer (19,20). Therefore, much effort to improve the survival of cancer patients also focus on the search for
natural compounds for treatment of cancer to escape TRAIL resistance or to sensitize cancer cells to TRAIL-induced apoptosis.

Induction of DR5 expression is an important mechanism underlying sensitization of TRAIL-mediated apoptosis (21-25). In this study, we failed to detect any induction of DR5 expression in BBR-treated Caki cells, indicating that DR5 modulation has a restricted role in BBR-induced sensitization to TRAIL-induced apoptosis in these cells. Several natural compounds such as triterpenoids, silibinin, and flavopiridol can induce downregulation of c-FLIP and subsequent sensitization to TRAIL-induced apoptosis in cancer cells (26,27). In our present study, treatment with BBR induced downregulation of c-FLIP and the transient expression of c-FLIP partly abrogated induction of apoptosis by combined treatment with BBR and TRAIL (data not shown). These results suggested a critical role of c-FLIP downregulation in mediating the augmentation of TRAIL-induced apoptosis by BBR. It is generally recognized that c-FLIP protein levels can be transcriptionally regulated through the NF-κB or c-Fos pathway (28,29), or by ubiquitin/proteasome-mediated degradation (30-32). In this study, we found that BBR promotes ubiquitin/proteasome-mediated degradation of c-FLIP, leading to downregulation of c-FLIP, but not by transcriptional control.

It was reported that BBR induced the ROS-mediated apoptosis and downregulated the expression of Bcl-2 protein in HepG2 cells, which were prevented by NAC treatment (17). We also found that treatment with BBR induced ROS generation and that pretreatment with NAC prevented BBR plus TRAIL-induced apoptosis. Contrary to this report, we failed to detect any change in Bcl-2 protein levels but found...
marked change of Mcl-1 protein levels by BBR treatment in our system. This controversial result might reflect the differences in the signaling pathways depending on the cellular contexts. Recently, several studies have shown that ROS downregulated c-FLIP or Mcl-1 levels and increased the sensitivity to apoptotic stimuli (33-35). Therefore, we investigated whether downregulation of c-FLIP and Mcl-1 was actually mediated by ROS signaling pathway. In the presence of NAC, the decreased levels of c-FLIP and Mcl-1 caused by BBR were restored. Taken together, BBR-stimulated TRAIL-induced apoptosis appears to be dependent on the formation of ROS for downregulation of c-FLIP and Mcl-1 proteins. BBR triggered a proteasomal proteolytic pathway which enhanced protein degradation such as HIF-1β degradation (36). Parallel to this observation, we observed that BBR triggered c-FLIP and Mcl-1 degradation through a proteasomal proteolytic pathway. However, further study is needed for the mechanistic study to elucidate BBR-induced activation of proteasomal signaling pathway.

In summary, our results provide the first mechanistic evidence that BBR treatment results in ROS-mediated downregulation of c-FLIP and Mcl-1, rendering cancer cells more sensitive to TRAIL. In addition, we suggest that BBR may be a potentially important therapeutic approach for enhancing sensitivity to TRAIL via the inhibition of anti-apoptotic proteins, such as c-FLIP and Mcl-1 in human renal cancer.

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