Original Paper

Berberine Induces Apoptosis in p53-Null Leukemia Cells by Down-Regulating XIAP at the Post-Transcriptional Level

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Key Words

Berberine • Apoptosis • Leukemia • XIAP

Abstract

Background: Berberine exerts anticancer activities both *in vitro* and *in vivo* through different mechanisms. However, the underlying molecular mechanisms of berberine induced p53-independent apoptosis remain unclear. **Methods:** The p53-null leukemia cell line EU-4 cells were exposed to berberine. Then the cell viability and apoptosis were determined. Western blot and PCR were employed to detect the expression of apoptosis related protein, XIAP and MDM2. Small interfering RNA (siRNA) was applied to knock down endogenous expression of MDM2 and XIAP. **Results:** Berberine induced p53-independent, XIAP-mediated apoptotic cell death in p53-null leukemia cells. Treatment with berberine resulted in suppression of XIAP protein in a dose- and time- dependent manner. Berberine induced down-regulation of XIAP protein involving inhibition of MDM2 expression and a proteasome-dependent pathway. Moreover, inhibition of XIAP by berberine or siRNA increased the sensitivity of leukemia cells to doxorubicin-induced apoptosis. **Conclusion:** Our findings characterize the molecular mechanisms of berberine-induced caspase activation and subsequent apoptosis, and berberine may be a novel candidate inducer of apoptosis in leukemia cells, which normally lack p53 expression.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most aggressive hematologic malignancy during infancy and childhood [1]. Modern multi-agent chemotherapy regimens have increased the cure rate of this disease, but the outcomes of patients who relapse remain

poor [2]. Chemoresistance is one of the major risk factors for relapse and poor treatment outcomes in childhood ALL [3]. However, further intensification of existing chemotherapy regimens is unlikely to increase the cure rate and may significantly increase the incidence of toxicities. Survivors of childhood ALL are at risk of multiple late effects related in part to the intensity of their therapy [4]. Therefore, novel anti-ALL agents must be developed to overcome chemotherapy resistance and reduce nonspecific toxicities.

As the best known member of the inhibitor of apoptosis protein (IAP) family, X-linked inhibitor of apoptosis protein (XIAP) is known as a promising therapeutic target for overcoming drug resistance in cancer cells. XIAP directly inhibits upstream caspase 9 and downstream caspases 3 and 7; thus, it controls the intrinsic and extrinsic pathways of apoptosis [5]. In addition, ubiquitination by the XIAP RING domain targets caspase 3 for degradation by the proteasome [6]. Mechanisms that regulate XIAP expression are complex. XIAP is transcriptionally regulated by the NF- κ B signaling pathway [7], and its expression is controlled by oncogenes such as MDM2 upon translation through the internal ribosome entry site (IRES), which is a unique sequence located in the 5' untranslated region of XIAP mRNA [8]. XIAP protein is also post-translationally regulated by ubiquitination of its own E3 ligase as well as E3 ligases from other IAPs [9]. XIAP activity is affected by interaction with specific endogenous inhibitors such as Smac/DIABLO, XAF-1, and HtrA2/Omi [10-12].

Berberine, a natural isoquinoline alkaloid derived from *Berberis* species, has multiple pharmacological properties, including anti-inflammatory, anti-diarrhea, and anti-oxidative activities [13]. Evidence of the anti-cancer properties of berberine has been obtained, and the alkaloid has been shown to exert anti-proliferative, cytotoxic, and pro-apoptotic activities in numerous cancer cell lines, including leukemia [14-16]. We have previously reported that the response of pediatric ALL cells to berberine-induced cytotoxicity and apoptosis is closely associated with both MDM2 expression levels and p53 status [17]. However, the underlying mechanism of the p53-independent anticancer action of berberine requires further clarification.

In this study, we demonstrate that berberine-induced caspase-dependent apoptosis was mediated by targeting XIAP. Berberine down-regulated XIAP at the post-transcriptional level, and the mechanism involved included MDM2 suppression and proteasomal degradation. Moreover, inhibition of XIAP enhanced cell death and increased the sensitivity of ALL cells to doxorubicin-induced apoptosis. These findings suggest that berberine may be a novel candidate inducer of XIAP-mediated caspase activation and apoptosis in leukemia cells, which normally lack p53 expression.

Materials and Methods

Cells and reagents

The EU-4 leukemia cell line was established from childhood ALL patients at Emory University. EU-4 cells lack p53 expression and express very low levels of MDM2. Authentication of cell line was performed by testing its immune phenotypes and cytogenetic profiles in prior publications [17-20]. EU-4 cells were grown in standard culture medium (RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 50 units penicillin, and 50 g/mL streptomycin) at 37 °C and 5% CO₂.

Berberine, actinomycin D, and MDM2 antibody were purchased from Sigma. XIAP (sc-55551), Bax, Bcl-xl, caspase 9, PARP, and ubiquitin (Ub) antibodies were purchased from Santa Cruz. Caspase 3, phospho-MDM2 (Ser166), and XIAP (3B6) antibodies were purchased from Cell Signaling. The concentrations of all antibodies were set according to the manufacturers' instructions. The caspase inhibitor Z-VAD-FMK, the protein synthesis inhibitor Cycloheximide (CHX), and the proteasome inhibitor MG132 were obtained from Beyotime.

Cytotoxicity assay

The cytotoxic effect of berberine on EU-4 cells was determined using Cell Counting Kit-8 (CCK-8, Dojindo) assay according to the manufacturer's instructions. Cells cultured in 96-well plates were treated

with different concentrations of berberine for a 70 h period. CCK-8 solution was subsequently added to the mixture and incubation continued for an additional 2 h before optical density (OD) reading using a microplate reader (BioTek) at 450 nm. Appropriate controls lacking cells were included to determine the background absorbance.

Apoptosis detection

Flow cytometry was performed to analyze the degree of apoptosis induced by berberine. Briefly, cells with or without berberine treatment were washed twice with PBS and stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (KeyGEN). FACS analysis was performed on a FACScan system (Becton-Dickinson) with CellQuest software (Becton-Dickinson), and 10,000 events were collected for each sample. To visualize the morphology of apoptotic cells, stained cells were deposited onto slides and analyzed by fluorescence microscopy (Nikon).

Pulse-chase assay

The degradation rate of XIAP mRNA was examined by a standard actinomycin D analysis. EU-4 cells were harvested and their total RNA was isolated at different times after treatment with 5 mg/mL actinomycin D in the presence or absence of berberine. The XIAP mRNA was detected by quantitative RT-PCR. XIAP protein turnover was assessed by a standard protein-synthesis inhibitor CHX assay. Cells were treated with 100 µg/mL CHX at different times before harvest in the presence or absence of berberine. Western blot analysis revealed the expression levels of XIAP.

Reverse transcription (RT)-PCR

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed with ReverTra Ace qPCR RT Master Mix (TOYOBO). Specific primers (5'-GGACTTCGAGCAAGAGATGG-3' and 5'-AGCACTGTGTTGGCGTACAG-3') were used to amplify actin cDNA for loading controls. XIAP cDNA fragments were amplified by primers 5'-ATGACTTTTAACAGTTTGAAGG-3' and 5'-GCTCGTGCCAGTGTGTGCTG-3'. MDM2 cDNA fragments were amplified by primers 5'-CATTGTCCATGGCAAAACAG-3' and 5'-GGCAGGGCTTATTCCTTTTC-3'. PCR products were separated on 1.5% agarose gels and stained with GoldView. Images were visualized under UV light and photographed using a Gene Snap imaging system. To confirm XIAP mRNA expression or turnover, quantitative PCR was performed with a 7900HT real-time PCR system (Applied Biosystems) using SYBR Green Realtime PCR Master Mix (TOYOBO). Quantity values for gene expression were generated by the relative quantification ($2^{-\Delta\Delta CT}$) method, in which the fluorescence generated by each sample was normalized to that of the actin product for each gene.

Western blot

Cells were lysed for 30 min at 4 °C in a buffer solution composed of 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% (v/v) Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 20 g/mL aprotinin, and 25 g/mL leupeptin. To detect the cellular localization of MDM2, a cytoplasmic fraction was isolated using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer's instruction. Equal amounts of protein extracts were resolved by SDS-PAGE. Following transfer to a polyvinylidene difluoride membrane (Millipore), the fraction was blocked for 2 h at room temperature with buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 5% non-fat milk and incubated overnight with specific antibodies at 4 °C. After incubation with HRP-labeled secondary antibody for 2 h at room temperature, the blots were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Caspase 3 activity assay

Caspase 3 activity was measured using the Caspase 3 Activity Assay Kit (Beyotime). Cell lysates were prepared after treatment according to the manufacturer's protocol. A mixture of 10 µL of the cell lysate, 80 µL of reaction buffer, and 10 µL of 2 mM caspase 3 substrate (Ac-DEVD-pNA) was placed in 96-well plate and incubated overnight at 37 °C. Samples were measured using microplate reader (BioTek) at 405 nm. Protein quantification was done using RC DC Protein Assay Kit (Bio-Rad). Caspase 3 activity was calculated as unit per mg protein.

Fig. 1. Apoptotic effects of berberine on EU-4 cells. (A) EU-4 cells were treated with different doses of berberine for 72 h, and cell viability was detected by CCK-8 assay. (B) EU-4 Cells were treated with 50 and 100 μM berberine for 72 h and then stained with Annexin V-FITC and PI. The apoptotic cells (Annexin V-FITC+, green) and dead cells (PI+, red) were examined by fluorescence microscopy. (C) EU-4 cells were treated with different doses of berberine for 72 h. Cells were stained with Annexin V-FITC and PI and quantitated by flow cytometry. (D) EU-4 cells were treated with different doses of berberine for 72 h, and expression of the indicated proteins was detected by Western blot. (E) EU-4 cells were treated with 100 μM berberine for the indicated times, and expression of the indicated proteins was detected by Western blot. Data represent the mean±SD of three independent experiments. ** $P < 0.01$ compared with the control.

RNA interference

To achieve RNA interference (RNAi), EU-4 cells were transfected with siRNA (Invitrogen) specific for human MDM2 and XIAP by HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocols. After 48 h, transfected cells were collected, lysed, and immunoblotted. The siRNA sequences used were as follows: MDM2, 5'-CAACATATTGTATATTGTT-3'; XIAP, 5'-GGTCAGTACAAAGTTGAAA-3.

Immunoprecipitation

To assess XIAP ubiquitination, EU-4 cells were treated with berberine. The proteasome inhibitor MG132 was added 4 h prior to cells harvest to inhibit proteasome-mediated degradation of ubiquitinated-XIAP. Equal volumes of cell lysate were incubated overnight with XIAP antibody at 4 °C, followed by incubation with 40 μL of Protein A+G Agarose beads (Beyotime) at 4°C with gentle rocking for 2 h. The immunoprecipitated complexes were washed at least four times with the lysis buffer. Finally, Ub expression was detected by Western blot.

Statistical analysis

Results are expressed as mean±SD. Statistical difference was determined by one-way ANOVA followed by SNK test for multiple comparisons. A probability value of $P < 0.05$ was considered statistically significant.

Results

Berberine induces potent apoptosis in p53-null ALL cells

The effects of berberine on cell viability were examined by CCK-8 assay. Berberine exhibited strong cytotoxic effects on EU-4 cells lacking p53 expression (Fig. 1A). The relationship between berberine-induced cell death and apoptosis induction was determined

Fig. 2. Berberine induces caspase-dependent apoptosis involving down-regulation of XIAP. (A) EU-4 cells were treated with and without 50 μ M Z-VAD-FMK for 30 min, followed by 100 μ M berberine. Cell apoptosis was detected by flow cytometry. (B) EU-4 cells were treated with different doses of berberine for 72 h. Caspase 3 activity was measured with the substrate Ac-DEVD-pNA using Caspase 3 Activity Assay Kit. (C) EU-4 cells were treated with different doses of berberine for 72 h, and expression of the indicated proteins was detected by Western blot. (D) EU-4 cells

were treated with 100 μ M berberine for the indicated times, and expression of the indicated proteins was detected by Western blot. Data represent the mean $\pm$ SD of three independent experiments. ** P < 0.01 compared with the control.

by cell staining with Annexin V-FITC and PI and subsequent fluorescence microscopy analysis. As shown in the upper panel of Fig. 1B, over 80% of the cells were Annexin V-FITC-positive after treatment with 50 μ M berberine. The bottom panel in Fig. 1B shows that the pro-apoptotic effect was stronger after treatment with 100 μ M berberine. Fig. 1C shows the quantities of apoptotic cells treated with different berberine doses, as detected by flow cytometry. Consistent with the CCK-8 results, berberine induced dose-dependent apoptosis in EU-4 cells. Western blot assay confirmed this result and determined the expression of Bcl-2 family proteins. Fig. 1D shows that berberine significantly affected Bcl-2 family proteins, such as Bax and Bcl-xl. We simultaneously evaluated the activation of caspases 3 and 9 as well as the cleavage of PARP during berberine treatment. As shown in Fig. 1E, berberine induced the activation of caspase 3, caspase 9, and PARP in a time-dependent manner. These results indicate that berberine induces p53-independent apoptosis.

Berberine induces caspase-dependent apoptosis involving down-regulation of XIAP

To further demonstrate whether or not berberine induced apoptosis is caspase-dependent, we pretreated EU-4 cells with a pan-caspase inhibitor Z-VAD-FMK and observed berberine-induced apoptosis. As shown in Fig. 2A, berberine-induced apoptosis was inhibited in the presence of Z-VAD-FMK. To investigate the significance of caspase 3 activity in berberine-induced apoptosis, we measured caspase 3 activity via a colorimetric assay based on the cleavage of the synthetic peptide Ac-DEVD-pNA. Treatment of EU-4 with berberine for 72 h resulted in a dose-dependent increase in caspase 3 activity (Fig. 2B). XIAP regulates caspase activity, which affects apoptosis. XIAP expression was further determined by Western blot. Berberine treatment down-regulated XIAP protein expression in a dose- and time-dependent manner (Fig. 2C and 2D).

Berberine treatment does not regulate XIAP mRNA expression levels

To clarify the underlying mechanisms of the down-regulation of XIAP protein expression by berberine, RT-PCR analysis was conducted to determine whether or not berberine directly decreases XIAP mRNA expression. Consistent with previous observations on other cancer cells, berberine did not show directly inhibitory effects on XIAP mRNA expression (Fig. 3A). Quantitative RT-PCR analysis was conducted to confirm these results. Quantitative RT-PCR showed that berberine treatment did not affect XIAP mRNA levels (Fig. 3B), which indicates that berberine treatment does not directly regulate XIAP transcription. Pulse-chase assay

Fig. 3. Berberine treatment shows no effect on mRNA levels of XIAP. (A) EU-4 cells were treated with 100 μ M berberine for the indicated times, followed by RT-PCR analysis. (B) XIAP mRNA expression was confirmed by quantitative RT-PCR after treatment. (C) EU-4 cells were treated with or without 100 μ M berberine for 24 h, followed by addition of 5 mg/mL actinomycin D. Cells were harvested at the indicated times and the amount of XIAP mRNA was determined by quantitative RT-PCR. Data represent the mean $\pm$ SD of three independent experiments.

Fig. 4. The effect of berberine-inhibited MDM2 on XIAP expression. (A) EU-4 cells were treated with different doses of berberine for 72 h. The expressions of total MDM2 and MDM2 phosphorylation at site 166 in whole cell extract (WCE) as well as MDM2 expression in cytoplasmic extract (Cyt) were analyzed by Western blot. (B) EU-4 cells were treated with 100 μ M berberine for the indicated times. Western blot showed the time course of MDM2 inhibition by berberine. (C) RT-PCR for MDM2 mRNA expression in EU-4 cells treated with 100 μ M berberine for the different times indicated. (D) EU-4 cells were transfected with different concentrations of either MDM2 siRNA or control siRNA for 48 h. Efficiency of siRNA in silencing endogenous MDM2 was detected by Western blot. (E) XIAP protein expression in EU-4 cells transfected with either control siRNA or MDM2 siRNA. ** P < 0.01 compared with the control.

was conducted to evaluate whether or not XIAP mRNA stability is affected by berberine treatment using actinomycin D (Fig. 3C). No difference in the degradation rate of XIAP mRNA was observed between berberine-treated and untreated EU-4 cells.

Berberine inhibits the protein synthesis of XIAP by down-regulating MDM2 expression

XIAP is a translational target of MDM2, which means inhibition of MDM2 by siRNA results in reduced translation and expression of XIAP [8]. The involvement of MDM2 in the regulation of XIAP after berberine treatment was investigated. The possible effects of berberine on MDM2 expression, particularly cytoplasmic MDM2 expression, were examined. Examination of whole cell extracts (WCE) of EU-4 cells showed that total MDM2 expression is inhibited and MDM2 phosphorylation at serine 166 is reduced following berberine

Fig. 5. Berberine down-regulates XIAP expression at the post-translational level. (A) CHX pulse-chase assay for detection of XIAP protein turnover in EU-4 cells treated with or without 100 μ M berberine for 24 h. The expression of the indicated proteins was detected by Western blot. (B) XIAP expression levels in the CHX pulse-chase assay were quantified by densitometry analysis and statistically analyzed from three independent experiments. Actin was used as an internal control. * $P < 0.05$ compared with the control. (C) EU-4 cells were pre-incubated with or without 100 μ M berberine for 20 h, followed by treatment with or without the proteasome inhibitor MG132 (20 μ M) at 4 h before harvesting. Ubiquitinated XIAP protein was immunoprecipitated using anti-XIAP antibody and further detected with ubiquitin antibody.

treatment (Fig. 4A). Phosphorylation of MDM2 at serine 166 is required for translocation of MDM2 from the cytoplasm to the nucleus [21]. Berberine regulates the translocation of MDM2 from the nucleus to the cytoplasm, which is required for the binding of MDM2 with the XIAP mRNA, to regulate XIAP translation. The cellular redistribution of MDM2 following berberine treatment in EU-4 cells was examined. Berberine treatment also decreased cytoplasmic MDM2 expression, as shown in Figure 4A. Berberine treatment suppressed the mRNA expression and protein expression of MDM2 in a time-dependent manner (Fig. 4B and 4C). Down-regulation MDM2 expression affects the protein synthesis of XIAP by RNAi to induce the degradation of MDM2. The efficiency of MDM2 siRNA in silencing endogenous MDM2 is shown in Fig. 4D. Inhibition of MDM2 by siRNA resulted in the reduced expression of XIAP, as shown in Fig. 4E. Down-regulation of MDM2 expression is thus involved in the inhibitory effect of berberine on XIAP expression.

Berberine down-regulates XIAP expression at the post-translational level

In response to apoptotic stimuli, XIAP is capable of auto-ubiquitination, which leads to proteasomal degradation [22]. To determine whether or not proteasomal degradation is involved in the down-regulation of XIAP protein after berberine treatment, the stability of XIAP protein was evaluated by pulse-chase assay (Fig. 5A). As shown in Fig. 5B, the half-life of XIAP protein in berberine-treated cells was less than that in untreated cells because of the susceptibility of the protein to degradation. Promotion of XIAP degradation by berberine through the ubiquitin-proteasome pathway was evaluated by immunoprecipitation and Western blot assay. As shown in Fig. 5C, XIAP was poly-ubiquitinated and the ubiquitinated protein accumulated in the cells following treatment with the proteasome inhibitor MG132. Berberine promoted the ubiquitination of XIAP, both in the presence and absence of MG132. Berberine thus reduces the stability of XIAP protein and promotes its ubiquitin-mediated proteasomal degradation.

Inhibition of XIAP expression is responsible for the apoptotic effect on EU-4 cells

Berberine treatment dramatically induced inhibitory activity associated with the down-regulation of XIAP expression, which results in the increased of cell apoptosis in a p53-independent manner. To test the contribution of XIAP down-regulation to the induction of apoptotic effects by berberine, we evaluated the effect of down-regulation of XIAP on EU-4 cells. We first examined the expression of MDM2 and XIAP in EU-4 cells treated with

Fig. 6. The effect of down-regulation of XIAP on EU-4 cells. (A) EU-4 cells were treated with different doses of doxorubicin for 48 h, and expression of the indicated proteins was detected by Western blot. (B) EU-4 cells were transfected with either XIAP siRNA or control siRNA and combined with berberine (100 μ M) or doxorubicin (1 μ M) for 48 h. Cell apoptosis was detected by flow cytometry. Data represent the mean $\pm$ SD of three independent experiments. ** $P < 0.01$ compared with the control. (C) EU-4 cells were transfected with different concentrations of either XIAP siRNA or control siRNA for 48 h. Efficiency of siRNA in silencing endogenous XIAP was detected by Western blot.

doxorubicin, a chemotherapeutic agent clinically used for ALL. Doxorubicin treatment for 48 h did not affect the expression of MDM2 and XIAP (Fig. 6A). To further explore whether or not berberine could increase the sensitivity of EU-4 cells to doxorubicin, EU-4 cells were treated with doxorubicin alone or cooperated with berberine for 48 h. As shown in Fig. 6B, treatment with doxorubicin alone had no significant effect on the apoptosis of EU-4 cells, as this cell line is generally resistant to doxorubicin-mediated apoptosis. The combination of doxorubicin and berberine induced a marked increase in apoptosis compared with the control groups (Fig. 6B). The involvement of XIAP down-regulation in increasing the sensitivity of EU-4 cells to doxorubicin was investigated using XIAP siRNA. The knockdown effect of XIAP siRNA was confirmed by Western blot (Fig. 6C). XIAP siRNA efficiently inhibited XIAP expression 48 h after transfection. Fig. 6B shows that siRNA-mediated knockdown of XIAP increased the sensitivity of EU-4 cells to doxorubicin-induced apoptosis. Moreover, apoptosis was observed in cells following treatment with XIAP siRNA as a single agent. XIAP down-regulation alone was sufficient to induce apoptosis in EU-4 cells. Thus, berberine induces apoptotic effect and increases the sensitivity of EU-4 cells to doxorubicin-induced apoptosis, at least in part, by down-regulating XIAP.

Discussion

Loss of p53 activity is usually linked with several specific landmarks such as defect in growth arrest or apoptosis after DNA damage [23]. The p53 status is also a key factor for the sensitivity to anticancer agents and multiple studies have focused on this subject [24]. In our previous study, berberine induced apoptosis in wt-p53/MDM2-overexpressing ALL cells (EU-1). In the present study, apoptotic cell death was clearly dependent on caspase activation induced by berberine in p53-deficient/MDM2-expressing ALL cells (EU-4). Berberine directly down-regulated XIAP protein expression, a critical obstacle to apoptosis induction, at the post-transcriptional level. Inhibition of MDM2 protein also attenuated XIAP expression, at least in part, at the translational level.

Post-transcriptional regulation of XIAP expression is suggested in several hematologic and non-hematologic malignancies [25, 26]. We did not detect a decrease in XIAP mRNA expression or instability of XIAP mRNA levels after berberine treatment (Fig. 3), which indicates that berberine mainly regulates XIAP expression at the post-transcriptional level in EU-4 cells.

The possible regulation of XIAP expression occurs during translational initiation, and involves an IRES motif of the XIAP mRNA, which enables cap-independent translation and is essential for the maintenance of XIAP protein expression during cellular stress [27]. The RING finger domain of MDM2 interacts with XIAP IRES mRNA. Cytoplasmic redistribution of MDM2 causes a significant increase in XIAP protein levels in leukemia cells overexpressing MDM2, resulting in apoptosis resistance [8]. These results provide a mechanism by which MDM2 plays a p53-independent role in regulating expression of the anti-apoptotic factor XIAP, and our previous and present study thus confirm that MDM2 regulates XIAP expression in a p53-independent manner in berberine-treated EU-4 cells.

Berberine induced dephosphorylation of MDM2 at serine 166, which could lead to inhibition of nuclear entry of MDM2 or an increase in MDM2 translocation from the nucleus to the cytoplasm. Total MDM2 expression and cytoplasmic MDM2 decreased following berberine treatment. Thus, berberine could decrease XIAP IRES activity, which results in the down-regulation of XIAP protein. Berberine also induced the cytoplasmic translocation of MDM2 and interestingly inhibited MDM2 expression at the transcriptional level in EU-4 cells, which differs from the result of our previous study on wt-p53/MDM2-overexpressing ALL cells. Inhibition of MDM2 by siRNA could induce a moderate down-regulation of XIAP expression. The mechanistic insights generated in this study suggest a two-way role for MDM2 in the regulation of XIAP IRES-dependent translation during cellular stress, further revealing the effect of IRES-stimulating proteins in cancer.

Decreased protein stability is an alternative mechanism for post-translational down-regulation of XIAP expression. Several mechanisms responsible for XIAP degradation, such as proteasome-mediated degradation [22], cleavage by caspase [28] or calpains [29], have been described. Berberine decreased the stability of XIAP protein by auto-ubiquitination and degradation. Similar to this result, our previous study also found that berberine down-regulates MDM2 specifically by enhancing MDM2 ubiquitination [17]. However, the mechanism by which berberine promotes the ubiquitin-mediated degradation of XIAP requires further investigation. Phosphorylation of XIAP by Akt protects XIAP protein from ubiquitination and results in XIAP protein stabilization [30]. Berberine inhibits cellular growth and promotes apoptosis by down-regulating the PI3K/Akt signaling pathway [31] and represses Akt protein expression by modulating the mRNA expression level and protein degradation of Akt [32]. Phosphorylation and subcellular distribution of MDM2 are regulated by the PI3K/Akt pathway [21]. Activation of PI3K and its downstream target Akt phosphorylates cytoplasmic MDM2 on serine 166. Although the signaling pathway involved in such activity was not tested in the present study, we hypothesize that berberine inhibits the activation of PI3K/Akt pathway to reduce the phosphorylation and subcellular distribution of MDM2 and induce ubiquitination and degradation of XIAP. Therefore, decreased Akt activity may contribute to the apoptosis induced by berberine in EU-4 cells.

Targeting XIAP using small molecular inhibitors or antisense oligonucleotides results in induction of apoptosis in several cell lines and primary cells of solid tumors and hematological malignancies [33-35]. By contrast, homeostasis of normal blood cells is not affected by XIAP inhibition, and no significant pathological results from XIAP knock-down in mice following XIAP inhibition have been observed [36]. Therefore, XIAP is a promising target for novel treatment strategies. XIAP inhibition is currently being tested in clinical studies for the treatment of resistant malignancies [37]. Our data indicate that XIAP inhibition could enhance induction of apoptosis in childhood ALL cells. Increased sensitivity of leukemic cells to other chemotherapeutics, such as doxorubicin, is a key therapeutic benefit of berberine. Our results highlight the importance of XIAP targeting by berberine in increasing the sensitivity of leukemic cells to agents that promote apoptotic death.

Inhibition or down-regulation of XIAP lowers the apoptotic threshold, thereby inducing apoptosis and/or enhancing the cytotoxic action of chemotherapeutic agents. Approaches targeting XIAP are still relevant in the search for potent antitumor strategies, although the vulnerability of tumor cells to XIAP inhibition seems to depend on the apoptotic stimuli applied [38]. Molecular approaches for targeting XIAP expression include antisense

oligonucleotides [39], endogenous inhibitors [40], and a small-molecule inhibitor of XIAP [41]. However, safety is the major limitation of the abovementioned approaches, and other concerns, such as poor stability and transfection efficiency, have been raised [42]. Therefore, identifying and exploring an effective anti-cancer drug that can down-regulate XIAP expression is highly significant. Berberine-induced down-regulation of XIAP is responsible for apoptosis in EU-4 cells, and berberine treatment or siRNA-mediated XIAP inhibition results in sensitization of EU-4 cells to doxorubicin-induced apoptosis. These results suggest that berberine-induced XIAP inhibition may be a promising novel approach for the treatment of ALL.

Berberine is currently orally administrated in clinical practice for diarrhea treatment. Research on the anti-cancer effects of berberine remains in pre-clinical phases. Numerous studies, including the present one, show that different concentrations of berberine induce apoptosis of various tumor cell lines *in vitro* [43]. However, berberine is believed to be poorly absorbed in the gut wall. Pharmacokinetic studies in humans reveal low plasma concentrations of berberine, well below the dose used to induce apoptotic cell death *in vitro* [44]. Considering these findings, effective berberine doses may not be easily achieved under physiological conditions through oral administration. Intravenous injection could be a potential alternative to induce the apoptotic effect [45]. In addition, structural modifications [46] or novel formulations [47] may be developed to increase the bioavailability of berberine in cancer tissues. On the other hand, the blood clearance of berberine is rapid, and its biotransformation in the liver occurs quickly and substantially, allowing for immediate circulation of the metabolites in the body, which would account for the trace plasma concentrations obtained [48, 49]. Further research is required to translate pre-clinical data into clinical practice and apply *in vitro* observation on the pro-apoptotic effects of berberine in a clinical setting.

In conclusion, we provide experimental evidence to show that the berberine-induced apoptosis in chemoresistant leukemia cells is p53-independent but mediated by the suppression of XIAP protein levels. Berberine-mediated suppression of XIAP occurs at the post-transcriptional level. Inhibition of MDM2 induces down-regulation of XIAP protein. Important insights into the molecular mechanism responsible for the pro-apoptotic effects of berberine were obtained. The valuable information in this study can be used in the design and synthesis of other novel derivatives for cancer treatment.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81100357).

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