Berberine enhances chemosensitivity to irinotecan in colon cancer via inhibition of NF-κB

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Abstract. Previous studies have shown that irinotecan (CPT-11) impairs chemotherapy-induced apoptosis by activating nuclear factor-κB (NF-κB) and a number of strategies have been employed to augment chemosensitivity through the suppression of NF-κB activation. Berberine, a botanical alkaloid, was reported to enhance chemosensitivity to 5-fluorouracil and doxorubicin by suppressing NF-κB activation. In the present study, the effect of berberine on CPT-11-induced apoptosis was investigated through the inhibition of NF-κB. Inhibition of NF-κB activation by p65 small interfering RNA was shown to potentiate apoptosis induced by CPT-11. Berberine suppressed CPT-11-induced NF-κB activation in a dose-dependent manner and enhanced chemosensitivity to CPT-11 by downregulating NF-κB activation of antiapoptotic genes, c-IAP1, c-IAP2, survivin and Bcl-xL. The current observations indicate that berberine inhibits NF-κB activation and may be used to enhance CPT-11-induced apoptosis in colon cancer.

Introduction

Chemotherapy remains an important approach for cancer therapy. However, development of drug resistance during chemotherapy constitutes a predominant challenge in cancer treatment (1). A previous study demonstrated that nuclear factor-κB (NF-κB) activation is one mechanism by which tumors become resistant to chemotherapeutic agents (2).

The NF-κB family contains five members: c-Rel, RelA (p65), RelB, p50 and p52. In its inactive state, NF-κB resides in the cytoplasm due to inhibitory binding by the IκBa protein. Upon activation, IκBa undergoes phosphorylation and ubiquitination-dependent degradation, leading to p65 nuclear translocation and binding to a specific consensus sequence in the DNA, which then activates transcription of target genes. Previous studies have shown that NF-κB may be activated by anticancer chemotherapeutic compounds in a number of cancer cell lines (3-5) and activation of NF-κB attenuates apoptosis by regulating inhibitors of apoptosis, including c-IAP1, c-IAP2, TRAF1, TRAF2, survivin and Bcl-xL (5-7). Thus, a number of strategies have been employed to enhance apoptosis induced by chemotherapeutic compounds via NF-κB inhibition.

Irinotecan (CPT-11) and its more active metabolite, SN38, are topoisomerase I inhibitors that are efficacious in the treatment of specific neoplasms, including colorectal cancer (8). CPT-11 treatment has been shown to lead to the activation of NF-κB in a variety of human colorectal cancer cell lines (7). Several methods of inhibiting NF-κB activation, including antisense oligonucleotides, proteasome inhibitors and p65 small interfering RNA (siRNA) have been shown to reverse inducible chemotherapy resistance to CPT-11 (9-13).

Berberine, a botanical alkaloid derived from a plant that is used traditionally in Chinese medicine, has been reported to exhibit multiple biological and pharmacological properties. Berberine has potential as a chemotherapy adjuvant due to its low toxicity and anticancer properties (14). A previous study demonstrated that berberine potentiates apoptosis induced by 5-fluorouracil (5-FU) and doxorubicin through suppression of NF-κB activation (15). However, the effect of berberine on CPT-11-induced apoptosis and the underlying mechanisms have not been investigated. In the present study, berberine was observed to suppress CPT-11-induced NF-κB activation in a dose-dependent manner and significantly enhanced the sensitization of HCT116 cells to CPT-11-induced apoptosis by downregulating the NF-κB target antiapoptotic genes, c-IAP1, c-IAP2, survivin and Bcl-xL. These results indicate that berberine enhances chemosensitivity to CPT-11 through the inhibition of NF-κB activation.

Materials and methods

Materials. CPT-11, berberine, sulforhodamine B (SRB), trichloroacetic acid (TCA), acetic acid, tumor necrosis factor

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(TNF)-α, anti-β-actin and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TRIzol and cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies against p65, c-IAP1, c-IAP2, survivin and Bcl-xL were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Secondary antibodies for western blotting were obtained from Amersham Biosciences Biotech. (Piscataway, NJ, USA). All other reagents were obtained from Sigma-Aldrich unless stated otherwise.

Transfection of siRNA. Human colon cancer HCT 116 cells were obtained from ATCC (Manassas, VA, USA) and were seeded at $2x10^5$ cells/ml in 6-well. Cells plates were grown to 50% confluency and transfected with double-stranded siRNA for NF-κB p65 target sequence (sense: 5'-CUUCCAAGUUCCUAUAGAAdTdT-3' and antisense: 3'-dTdTGAAGGUUCAAGGAUAUCUU-5') or with a siRNA nonspecific control (Guangzhou Ribobio Co. Ltd., Guangzhou, China). Silencing was confirmed by the electrophoretic mobility shift assay (EMSA) and western blotting.

SRB assay. Cytotoxicity was determined by the SRB assay as described previously (16). Cells were seeded into 96-well plates and exposed to various concentrations of berberine with or without CPT-11. Following 48-h incubation, cells were fixed with TCA for 1 h at 4˚C, air-dried and stained with 0.4% SRB solution for 30 min at room temperature. Following staining, the SRB solution was removed and cells were subsequently washed with 1% acetic acid five times. Tris base solution (10 mM; pH 10.5) was used to dissolve the protein-bound dye and the plate was agitated on a plate shaker (Nangjing Changxiang Co. Ltd., Nangjing, China) for 10 min. The OD_{570} was determined using a 96-well plate reader (MRX; Dynex Technologies, Chantilly, VA, USA).

Western blotting. HCT116 cells were seeded into 6-well plates and exposed to the indicated concentrations of berberine with or without CPT-11. Following treatment, cells were harvested using lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM β-glycerophosphate and 1:1,000 protease inhibitors]. Protein concentrations were determined by the bicinchoninic acid method. Total protein (25 μg) was separated using 8-12% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose blotting membranes. Monoclonal antibodies against p65 (1:2,000), c-IAP1 (1:500), c-IAP2 (1:500) or β-actin (1:8,000) were used. Immunopositive bands were visualized using the Amersham ECL™ Plus Western Blotting Detection kit (GE Healthcare, Piscataway, NJ, USA).

EMSA. EMSA was performed as previously described (17,18). Biotin 3' end-labeled DNA probes containing the NF-κB consensus site, 3'-TCAACTCCCCTGAAAGGGTCCG-5' and 5'-AGTTGAGGGGACTTTCCCAGGC-3', were purchased from Invitrogen Life Technologies. EMSA was performed using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). NF-κB activation induced by TNF-α is considered as a positive control. Cells were treated with 20 ng/ml TNF-α for 30 min, TNF-α for 30 min plus 100X unlabeled NF-κB probes or cells were exposed to unlabeled NF-κB probes. Briefly, the nuclear proteins were incubated in 1X binding buffer, 50 ng/μl poly(dI-dC), 0.05% NP-40, 5 mM $MgCl_2$, 50 mM KCl, 2.5% glycerol and ddH_2O for 20 min at room temperature in a total volume of 20 μl. The reaction mixture was separated in a 6% nondenaturing polyacrylamide gel and transferred to a positively charged nylon membrane. The membrane was cross-linked and the biotin-labeled DNA was detected by chemiluminescence.

Hoechst 33258 staining. Hoescht 33258 staining was performed as previously described (19). Cells were treated with different concentrations of berberine (2.5-10 μM) for 48 h with or without 20 μM CPT-11 for 24 h and the cells were fixed with fixation fluid and washed with phosphate-buffered saline (PBS). Subsequently, Hoechst 33258 was used to stain the cells for 5 min and the cells were then washed with PBS three times. Cell apoptosis was observed by fluorescent microscopy (Olympus, Tokyo, Japan).

Assessment of apoptotic and necrotic cell death. Apoptotic and necrotic cell death was assessed as previously described (20,21). The effect of berberine on CPT-11-induced apoptosis was evaluated by Annexin V/propidium iodide (PI) double staining and flow cytometry. HCT-116 cells were incubated with 10 μM berberine for 48 h and/or 20 μM CPT-11 for 24 h. Cells were harvested and resuspended in binding buffer [10 Mm HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) followed by Annexin V-flurescein isothiocyanate (FITC) and PI. The percentages of viable, early apoptotic, late apoptotic and necrotic cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Statistical analysis. Statistical analysis between groups was performed by unpaired Student's t-test with SigmaPlot 10.0 software (Systat Software, Inc., San Jose, CA, USA). Data are presented as the mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of CPT-11 on cell viability in HCT116 cells. The SRB assay was used to detect the cytotoxic effects of CPT-11 in the HCT116 colon cancer cell line. Cells were treated with CPT-11 for 24 h at concentrations of 5, 10, 20, 40 μM. Fig. 1 shows that CPT-11 did not affect the viability of HCT116 cells below 40 μM.

Inhibition of NF-κB activation enhances chemosensitivity to CPT-11 in HCT116 cells. It has been shown that inducible resistance to CPT-11 may be reversed by inhibiting NF-κB (9-13). To confirm this effect, p65 siRNA was used in the present study. HCT116 cells were transfected with p65 siRNA for 48 h, followed by treatment with 20 μM CPT-11 for 2 h. Western blot analysis confirmed that the expression of p65 was reduced in cells treated with p65 siRNA relative to their untreated counterparts and siRNA negative controls (Fig. 2A). Consistent with the downregulation of p65 expression, NF-κB

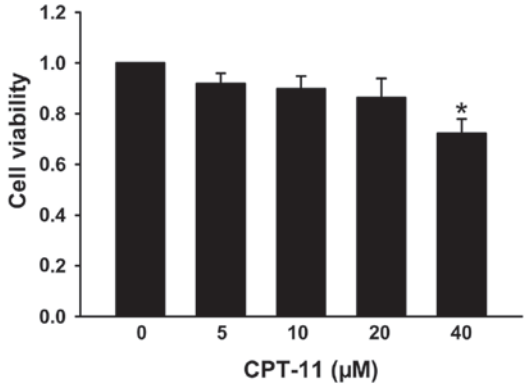

Figure 1. Effect of CPT-11 on cell viability in HCT116 cells. Cells were treated with various concentrations of CPT-11 (5-40 μM) for 24 h and the SRB assay was used to observe cell viability (n=4; *P<0.05, vs. control cells not exposed to CPT-11). CPT-11, irinotecan; SRB, sulforhodamine B.

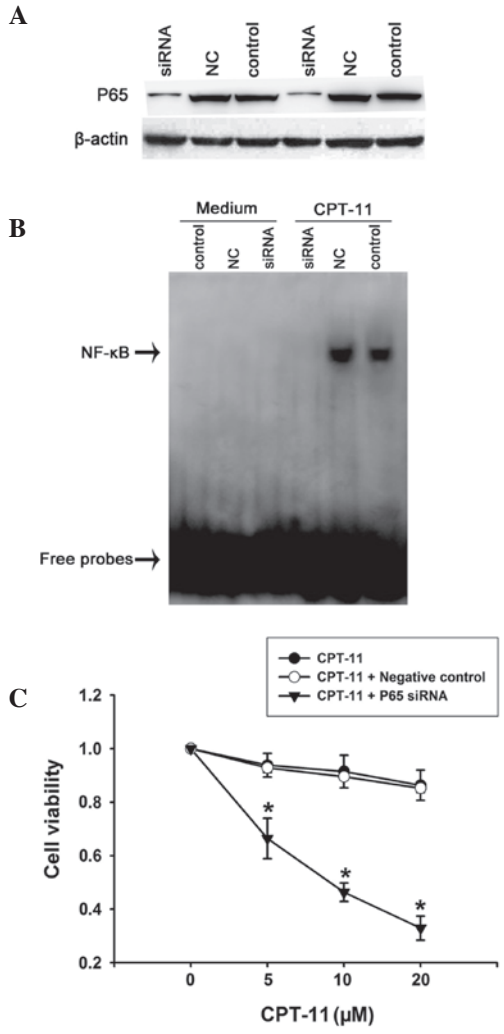

Figure 2. p65 siRNA enhances chemosensitivity to CPT-11 in HCT116 cells. Cells were transfected with p65 siRNA (siRNA, 100 nM) or an NC (100 nM) for 48 h, followed by treatment with 20 μM CPT-11 for 2 h. (A) Western blot analysis was used to detect the effect of p65 siRNA on NF-κB p65 expression. (B) Nuclear extracts were analyzed for NF-κB activation by EMSA. (C) p65 siRNA enhanced chemosensitivity to CPT-11. Cells were transfected with p65 siRNA for 48 h and then exposed to CPT-11 (5-20 μM) for 24 h. Cell viability was observed by the SRB assay (n=4; *P<0.05, vs. CPT-11 + negative control group). siRNA, small interfering RNA; CPT-11, irinotecan; NC, negative control; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; .SRB, sulforhodamine B.

activation was shown by EMSA to be significantly inhibited by the siRNAs (Fig. 2B).

To address whether the inhibition of NF-κB enhances chemosensitivity to CPT-11 in HCT116 cells, the effect of p65 siRNA on cell viability of CPT-11-treated cells was determined. Following 48-h transfection with p65 siRNA, HCT116 cells were incubated with CPT-11 (5-20 μM) for 24 h. The siRNA studies showed that p65 siRNA suppresses CPT-11-induced NF-κB activation and enhances the chemosensitivity to CPT-11 in HCT116 cells (Fig. 2C). This is in agreement with the hypothesis that inducible resistance to CPT-11 may be reversed by inhibition of NF-κB.

Berberine enhances chemosensitivity to CPT-11 in HCT116 cells. The results in Fig. 2 confirmed that the inhibition of NF-κB reverses inducible resistance to CPT-11. Berberine has been reported to inhibit NF-κB activation in several cell lines (15). Thus, the effect of berberine on the cytotoxicity of CPT-11 in HCT116 cells was investigated.

The effect of berberine on the cell viability in HCT116 cells was observed. Fig. 3A shows that berberine had no effect on cell viability up to a concentration of 20 μM; thus, the non-cytotoxic concentrations of berberine, between 2.5 and 10 μM were used in the subsequent expriments. To observe the effect of berberine on the cytotoxicity of CPT-11 cells, cells were incubated with berberine (2.5-10 μM) for 48 h, followed by incubation with 20 μM CPT-11 for 24 h. The results in Fig. 3B demonstrated that the chemosensitivity to CPT-11 was enhanced by berberine, leading to a significant increase in the inhibition rate from 11.42±2.3% (20 μM CPT-11 alone) to 49.06±3.8% (10 μM berberine plus 20 μM CPT-11). Cell apoptosis was detected using Hoechst 33258 and Annexin V/PI staining and the results were consistent with the SRB assay. As shown in Fig. 3C, combination treatment of berberine and CPT-11 in HCT116 cells resulted in an increased number of cells with condensed and fragmented nuclei than CPT-11 or berberine treatment alone. Similar results were confirmed by Annexin V/PI staining (Fig. 3D). These results indicate that berberine enhances the sensitization of HCT116 cells to CPT-11-induced apoptosis.

Berberine inhibits NF-κB activation induced by CPT-11. Previous studies have shown that the NF-κB pathway is activated in HCT116 cells in response to CPT-11 treatment (7) and that colon cancer cells become more sensitive to CPT-11 by abrogating this activation (9-13). Thus, the results in Fig. 3 indicated that berberine may enhance the cytotoxicity of CPT-11 through the suppression of NF-κB activation. EMSA was used to determine the effect of berberine on CPT-11-induced NF-κB activation. HCT116 cells were pretreated with varying concentrations of berberine (2.5-10 μM) for 48 h and exposed to 20 μM CPT-11 for 2 h. Fig. 4 shows that berberine alone had no effect on NF-κB activation, but it suppressed CPT-11-induced NF-κB activation in a dose-dependent manner in HCT116 cells. These results indicate that berberine effectively blocks the activation of NF-κB induced by exposure to CPT-11 and enhances chemosensitivity to CPT-11 in HCT116 cells.

Berberine decreases c-IAP1, c-IAP2, survivin and Bcl-xL expression induced by CPT-11. As NF-κB regulates antiapop-

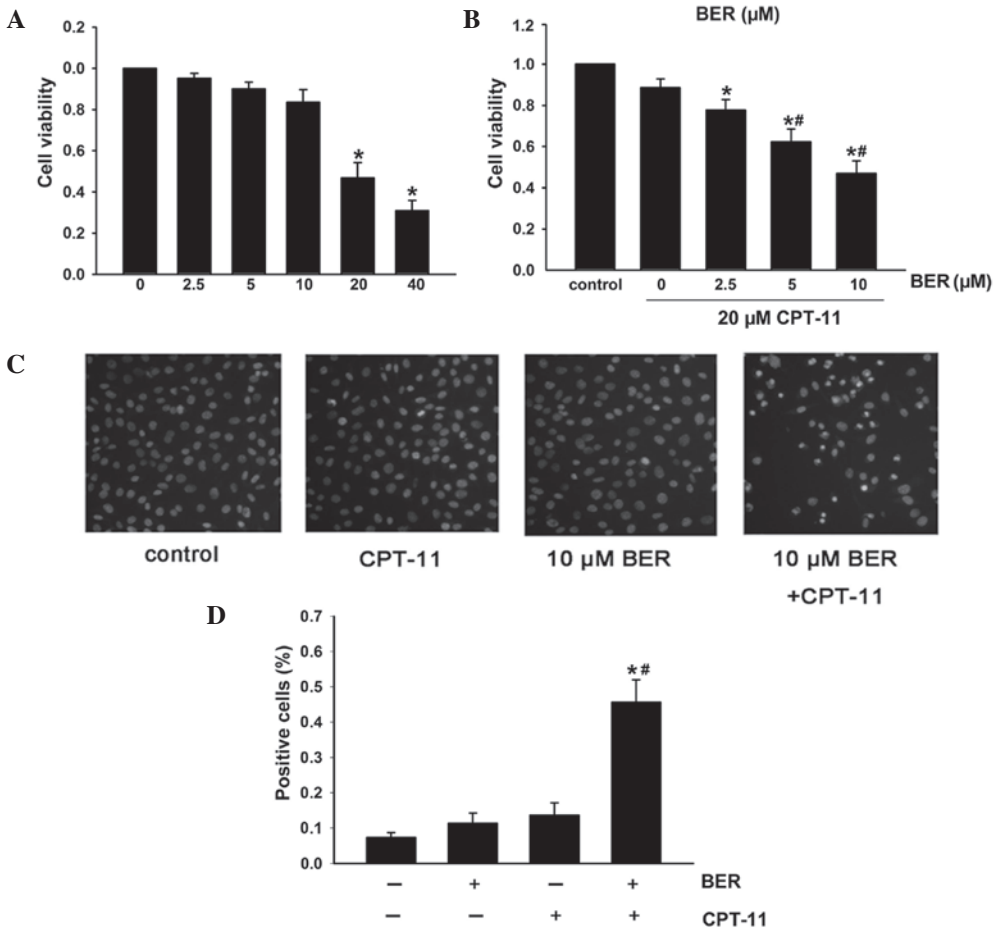

Figure 3. BER enhances chemosensitivity to CPT-11 in HCT116 cells. (A) Effect of berberine on cell viability in HCT116 cells. Cells were treated with various concentrations of BER (2.5-40 μM) for 48 h and the cell viability was detected by an SRB assay (n=5; *P<0.05, vs. control cells not exposed to berberine). (B) Cells were pretreated with BER (2.5-10 μM) for 48 h and then exposed to 20 μM CPT-11 for 24 h. The SRB assay was used to observe cell viability (n=5; *P<0.05, vs. control group; #P<0.05, vs. CPT-11 group). (C and D) Cells were incubated with 10 μM BER for 48 h, followed by incubation with 20 μM CPT-11 for 24 h. Apoptosis was detected by Hoechst 33258 and Annexin V/propidium iodide staining (n=3; *P<0.05, vs. cells not exposed to drugs; #P< 0.05 vs. CPT-11 group). BER, berberine; CPT-11, irinotecan; SRB, sulforhodamine B.

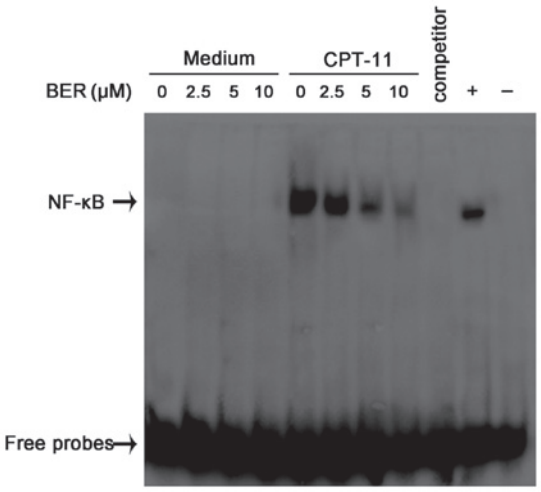

Figure 4. BER suppresses CPT-11-induced NF-κB activation. HCT116 cells were pretreated with BER (2.5-10 μM) for 48 h and exposed to 20 μM CPT-11 for 2 h. Nuclear extracts were assayed for NF-κB activation by EMSA. NF-κB activation induced by TNF-α is considered as a positive control. Cells were treated with 20 ng/ml TNF-α for 30 min (lane +) or TNF-α for 30 min plus 100X unlabeled NF-κB probes (lane competitor) or cells were exposed to unlabeled NF-κB probes (lane -). BER, berberine; CPT-11, irinotecan; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; TNF-α, tumor necrosis factor-α.

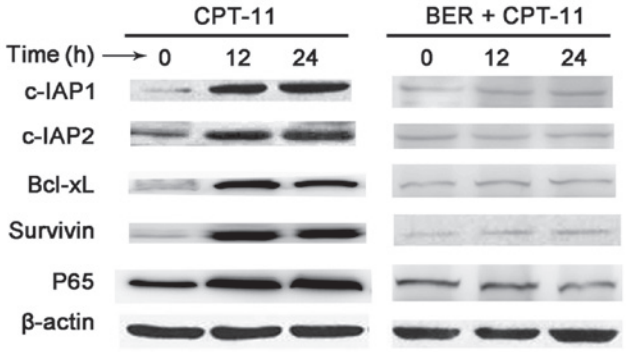

Figure 5. BER treatment decreased c-IAP1, c-IAP2, survivin and Bcl-xL expression induced by CPT-11. Cells were exposed to CPT-11 (20 μM) for 12 or 24 h and pretreated with or without berberine (BER, 10 μM) for 48 h. The expression levels of c-IAP1, c-IAP2, survivin, Bcl-xL and P65 were determined by western blotting. BER, berberine; CPT-11, irinotecan.

totic proteins, including c-IAP1, c-IAP2, survivin and Bcl-xL, the effect of berberine on CPT-11-induced expression of these antiapoptotic proteins was observed. CPT-11 was observed to induce the expression of the antiapoptotic proteins c-IAP1, c-IAP2, survivin and Bcl-xL (Fig. 5) and berberine treatment

blocked the expression of these proteins. This result indicates that berberine increases the cytotoxicity of CPT-11 by downregulating NF-κB target of antiapoptotic genes.

Discussion

Consistent with previous studies, the results of the current study showed that berberine enhances chemosensitivity to CPT-11 in HCT116 cells by inhibiting NF-κB activation. Berberine suppressed CPT-11-mediated NF-κB activation in a dose-dependent manner and potentiated CPT-11-induced apoptosis by downregulating NF-κB antiapoptotic target genes.

CPT-11 is widely used to treat certain neoplasms, including colorectal cancer. However, CPT-11 has been reported to lead to the activation of NF-κB in a variety of human colorectal cancer cell lines (7). When activated, NF-κB increases the expression of antiapoptotic genes, which promote cell survival and block apoptosis. In addition, colorectal cancer cells tend to be resistant to CPT-11 (5-7). A number of strategies of NF-κB inhibition, including antisense oligonucleotides, proteasome inhibitors and p65 siRNA, have been shown as promising approaches for enhancing CPT-11-induced apoptosis (9-13). In the present study, inhibition of NF-κB by p65 siRNA transfection was confirmed to significantly enhance chemosensitivity to CPT-11 in HCT116 cells. In addition, to the best of our knowledge, the current study is the first to show that berberine suppresses CPT-11-induced NF-κB activation in a dose-dependent manner in HCT116 cells. The results of the SRB assay, Hoechst 33258 and Annexin V/PI staining demonstrated that berberine potentiates CPT-11-induced apoptosis. Compared with previous methods, including the aformentioned methods, berberine is a simple, available and applicable means to increase CPT-11-induced apoptosis. The safety of berberine in humans has already been established and it is widely used as a therapeutic drug for the treatment of intestinal infections and diarrhea.

NF-κB-mediated antiapoptosis is essentially dependent on the ability of NF-κB to enhance transcription of target genes (22,23), including c-IAP1, c-IAP2, survivin and Bcl-xL, which prevents tumor cell killing effects (22-24). Thus, in the present study, western blotting was used to detect the effect of berberine on expression levels of these antiapoptotic proteins. The results demonstrate that berberine abolished CPT-11-induced expression of c-IAP1, c-IAP2, survivin and Bcl-xL (Fig. 5). Therefore, berberine may potentiate CPT-11-induced apoptosis by downregulating the NF-κB antiapoptotic target genes.

Myelosuppression and diarrhea are the two major toxicities associated with CPT-11 treatment and numerous studies have investigated pharmacological modulation to reduce CPT-11 toxicity. In the current study, concentrations between 2.5 and 10 μM berberine were non-toxic for HCT116 cells while still enhancing chemosensitivity to CPT-11. Therefore, CPT-11-induced toxicity may be alleviated by berberine treatment, thus, potentiating anticancer effects with lower doses of CPT-11. These results are consistent with those in previous studies, indicating that berberine is a non-toxic chemoadjuvant that sensitizes tumor cells to CPT-11 by inhibiting NF-κB. Future studies are likely to include *in vivo* studies to reduce CPT-11 toxicity using berberine as a chemoadjuvant to CPT-11.

By investiagting the mechanism of interplay between berberine, NF-κB and the cytotoxicity of CPT-11, the present study provides a basis for the rational choice of natural compounds, including berberine, in chemotherapy. In addition, the importance of basic cell biology in developing an integrated approach to pharmacological intervention is highlighted. The current study also indicates the possibility that natural compounds, which inhibit NF-κB activation, may be used to augment the cytotoxicity of chemotherapeutic agents.

Acknowledgements

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