Induction of Noxa Sensitizes Human Colorectal Cancer Cells Expressing Mcl-1 to the Small-Molecule Bcl-2/Bcl-\(x_L\) Inhibitor, ABT-737

Kenji Okumura, Shengbing Huang, and Frank A. Sinicrope

Abstract

**Purpose:** The intrinsic drug resistance of colorectal cancers is related in part to overexpression of prosurvival Bcl-2 family proteins. We determined the effects of ABT-737, a small-molecule inhibitor of Bcl-2/Bcl-x\(_L\) but not Mcl-1, on apoptosis induction alone and in combination with CPT-11 and explored mechanisms underlying their cooperativity.

**Experimental Design:** Human colorectal carcinoma cell lines (HCT116 wild-type and \(\text{Bax}^{-/-}\), HT-29, and RKO) were incubated with ABT-737 alone and combined with CPT-11 or bortezomib, and cell viability, caspase cleavage, and Annexin V labeling were measured. In drug-treated cell lines, protein-protein interactions were analyzed by immunoprecipitation. Lentiviral short hairpin RNA was used to knockdown Noxa expression.

**Results:** ABT-737 induced apoptosis in a dose-dependent manner and its coadministration with the topoisomerase I inhibitor, CPT-11, resulted in a synergistic cytotoxic effect. Apoptosis induction by the drug combination was associated with enhanced caspase-8, caspase-9, and caspase-3 activation and poly(ADP-ribose) polymerase cleavage that were completely abrogated in \(\text{Bax}^{-/-}\) knockout cells. ABT-737 unsequestered the BH3-only protein Bim from its complex with Bcl-\(x_L\) or Bcl-2 and disrupted the interaction of Bcl-\(x_L\) with Bak. CPT-11 treatment up-regulated Noxa expression, as did bortezomib, and enhanced Noxa/Mcl-1 complexes. CPT-11 also disrupted the Mcl-1/Bak interaction. Knockdown of Noxa using short hairpin RNA lentiviral constructs was shown to significantly attenuate the cytotoxic effect of CPT-11 or bortezomib combined with ABT-737 and inhibited caspase-3 cleavage.

**Conclusions:** Induction of Noxa by CPT-11 or bortezomib can sensitize colorectal cancer cells expressing Mcl-1 to ABT-737. Up-regulation of Noxa may therefore represent an important strategy to enhance the therapeutic efficacy of ABT-737 against colorectal cancer and other solid tumors.

Colorectal cancer is the third most common cancer and second leading cause of cancer-related mortality in the United States (1). This malignancy displays intrinsic apoptosis resistance related to the overexpression of prosurvival Bcl-2 proteins (2). Accordingly, new drugs are needed to circumvent Bcl-2-mediated resistance and to increase therapeutic efficacy. Proapoptotic BH3-only proteins are sensors of cellular stresses, including chemotherapeutic drugs, which bind to the hydrophobic cleft in prosurvival Bcl-2 family proteins to neutralize them, thereby shifting the balance in favor of proapoptotic molecules. Disabling prosurvival Bcl-2 proteins removes their restraint of downstream Bax and Bak. At least eight BH3-only members have been identified and these include Bad, Bid, Bim, Bmf, Hrk, Noxa, and Puma (3). The BH3-only proteins can be further divided into two subclasses: “activators” (e.g., Bim and tBid), which directly activate Bax/Bak to induce mitochondrial outer membrane permeabilization, and “sensitizers” (e.g., Bad, Bik, Bmf, Hrk, Noxa, and Puma), which do not activate Bax/Bak directly but instead neutralize prosurvival proteins (4). Studies indicate that BH3-only proteins bind promiscuously or selectively to prosurvival Bcl-2 proteins (5–7). Bim and Puma have been shown to target all prosurvival proteins and, accordingly, are more potent inducers of apoptosis in vitro than are Bad and Noxa which target only a subset (6).

Recently, BH3 mimetics have been developed as a new and novel class of anticancer drugs. ABT-737 is a BH3 mimetic and potent small-molecule antagonist that binds with high affinity to Bcl-2, Bcl-x\(_L\), and Bcl-w but not Mcl-1 (8). ABT-737 has been shown to lower the apoptotic threshold for certain chemotherapeutic agents and also showed impressive preclinical activity against lymphoma in a murine model (8, 9). ABT-737 has...
shown single-agent activity against leukemia, lymphoma, and small cell lung cancer in preclinical models where high levels of Bcl-2 and/or Bcl-xL and low levels or absent Mcl-1 were found (10–12). Given that ABT-737 binds to Mcl-1 with low affinity, tumor cell Mcl-1 expression has been shown to represent an important mechanism of resistance to this agent (10, 11, 13).

Colorectal cancers have been shown to frequently express prosurvival members of the Bcl-2 protein family (2). Although sparse data exist as to the activity of ABT-737 against solid tumor cell lines, it is likely that significant antitumor efficacy against colorectal cancers may require ABT-737 in combination with cytotoxic chemotherapy (12). CPT-11 (irinotecan) is widely used for the treatment of advanced colorectal cancer in humans. CPT-11 is a camptothecin derivative and DNA topoisomerase I inhibitor that is metabolized by carboxylesterases in vivo to SN-38 and is believed to block DNA transcription and replication (14). However, apoptosis resistance related to prosurvival Bcl-2 proteins (15) and p53 inactivation (16) limit the therapeutic efficacy of CPT-11, resulting in treatment failure and disease progression. Therefore, strategies to enhance the apoptotic susceptibility of tumor cells to CPT-11 and other chemotherapeutic agents is a major objective of cancer treatment.

In this study, we determined the ability of ABT-737 to enhance CPT-11-mediated cell killing in human colorectal cancer cells. We found that the ABT-737 and CPT-11 produced a synergistic cytotoxicity that was due to a Bax-dependent induction of apoptosis. Specifically, ABT-737 released Bim from its sequestration by Bcl-2 or Bcl-xL and released Bak from Bcl-xL. Furthermore, CPT-11 treatment markedly induced Noxa expression, which complexed with Mcl-1, and was associated with Bak release from Mcl-1.

**Materials and Methods**

**Cell culture, drugs, and reagents.** Human colorectal cancer cell lines HCT116, HT-29, RKO, and HCT116 Bak−/− knockout cells (gift of Dr. B. Vogelstein, Johns Hopkins University) were used. Cell lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum and with 1% penicillin/streptomycin, 10 mmol/L HEPES, and 1% sodium pyruvate. ABT-737 (Abbott), CPT-11 (Sigma), SN-38 (United States Biological), or bortezomib (PS-341; Millennium) were dissolved in DMSO to produce 20 or 10 mmol/L stock solutions that were aliquoted and stored at -20°C.

**Cell viability assay.** Cell viability was determined in the presence or absence of drug treatment using the MTS reduction assay per the manufacturer’s protocol (Promega), as previously described (17). To exclude interference by CPT-11 or SN-38 in the MTS assay, we added each drug individually in the absence of cells and showed no change in absorbance.

**Annexin V staining.** After drug treatment, adherent cells were detached from culture dishes by treating with Accutase (Innovative Biotechnology) for 5 to 15 min and combined with floating cells. Total cells were then washed with cold PBS twice and resuspended in 1× Annexin V binding buffer (BD Biosciences) at a concentration of 1×10^6 to 1×10^7 cells/mL. A single-cell suspension (100 μL) was stained with 10 μL Annexin V-FITC (BD Biosciences) for 15 min at room temperature in the dark. Propidium iodide (10 μL; Sigma) and 1× Annexin V binding buffer (380 μL) were then added to each tube. Two-color (FL1 and FL2) flow cytometry analysis was then done on a FACScan (Becton Dickinson). A minimum of 10,000 cells per sample were analyzed.

**Immunoprecipitation and Western blotting.** Mock-treated or drug-treated cells were harvested by scraping and then washed in cold PBS. After cell lysis, the cell pellet was resuspended in CHAPS buffer [5 mmol/L MgCl₂, 137 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% CHAPS, 10 mmol/L HEPES (pH 7.5)] for 30 min on ice with protease inhibitors (5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin). The cell lysate was subjected to IP as previously described (17). The antibodies used were as follows: Bim (Santa Cruz Biotechnology), Bak (Upstate Biotechnology), and Mcl-1 (BD Biosciences). Western blotting was performed as previously described (17). The antibodies used were as follows: Bcl-2 (BD Biosciences); Bcl-xL (Cell Signaling); Mcl-1 (BD Biosciences); Bax (Cell Signaling); Bak (Upstate Biotechnology); β-tubulin (Sigma); caspase-8 (BD Biosciences); Bid, caspase-9, and cleaved caspase-3 (all from Cell Signaling); poly(ADP-ribose) polymerase (Biomol); Bim (Santa Cruz Biotechnology); Puma (Cell Signaling); Noxa (Calbiochem); and p53 (Cell Signaling).

**Knockdown of Noxa using lentiviral short hairpin RNA.** Target sequences for Noxa and a control sequence were selected and short hairpin RNA (shRNA) template oligonucleotides (synthesized by the Mayo Clinic Molecular Biology Core Facility) were ligated into the lentiviral shRNA cloning and expression vector pSIH1-H1 (System Bioscience) using a quick ligation kit (New England Biolabs). Insertion sequence at the intended site was confirmed by sequencing. The control shRNA sequence was AATACGCACTAAACACATCAA (18). The targeting sequences for Noxa were GTAATTATTGACACATTTCTT (Noxa) and AATAGCGACTAAACACATCAA (18). The targeting sequences for Noxa were GTAATTATTGACACATTTCTT (Noxa) and AATAGCGACTAAACACATCAA (18). For lentivirus production, 2 μg endotxin-free lentiviral shRNA expression construct DNA was mixed with 20 μg lentivector packaging plasmid DNA mix (System Bioscience) and diluted in 400 μL serum reduction medium (Opti-MEM; Invitrogen) containing 20 μL Plus reagent (Invitrogen). After incubation at room temperature for 15 min, 30 μL Lipofectamine reagent mixed with 400 μL Opti-MEM was added dropwise into the above DNA/Plus complex and incubated for another 15 min. The lentivirus producer cell line 293T was transfected with the DNA/Lipofectamine/Plus complex overnight in Opti-MEM in the 5% CO₂ incubator at 37°C. The next day, the medium was replaced with fresh DMEM containing 2% heat-activated fetal bovine serum and incubation at 37°C continued. At 48 h post-transfection, the supernatants were collected, clarified, and filtered through Millipore filters. The supernatants were concentrated by adding 10% (final concentration) of PEG-8000 (Sigma), incubating
at 4°C overnight for no less than 12 h, and centrifuged at 1,500 × g for 10 min at 4°C. For transduction of the lentiviral shRNA expression construct (packaged in pseudotyped viral particles) into target cells, the growth medium of target cells was replaced with Opti-MEM containing 8 μg/mL polybrene (Sigma) and appropriate amounts of lentivirus. The cells were incubated overnight at 37°C. The medium was replaced with normal growth medium the following day. The efficiency of knockdown was tested 72 h post-transduction. Alternatively, 2 to 4 μg/mL puromycin (Sigma) was added at 48 h post-transduction and the puromycin-resistant pool of cells was used for subsequent experiments.

Calculation of combination index. The effect of combination between ABT-737 and CPT-11 at a fixed ratio (1:1) was analyzed using CalcuSyn software (Biosoft) as reported previously (21).

Statistical analysis. The values shown represent the mean ± SD for triplicate experiments. The statistical significance of the differences between experimental variables was determined using the Student’s t test. P < 0.05 was considered statistically significant.

Results

ABT-737 and CPT-11 cooperatively enhance cytotoxicity due to apoptosis. Treatment of HCT116 and HT-29 cell lines with ABT-737 (5-10 μmol/L) alone produced a modest reduction in cell viability. Furthermore, coadministration of ABT-737 (5-10 μmol/L) and CPT-11 (2.5-10 μmol/L) was shown to reduce cell viability to

Fig. 1. ABT-737 and CPT-11 cooperatively enhance cytotoxicity. Cultured HCT116 (A) and HT-29 (B) human colorectal cancer cell lines were incubated with ABT-737 and/or CPT-11 for 48 h at indicated doses. Cell viability was determined using the MTS reduction assay. Experiments were conducted in triplicate. Mean ± SD. HCT116 (C) and HT-29 (D) cells were treated with increasing concentrations of ABT-737 or CPT-11 for 48 h and the cell viability was determined using the MTS assay. IC_{50} values for ABT-737 or CPT-11 were calculated. Cells were treated with ABT-737 and CPT-11 at a fixed ratio (1:1), cell viability was measured, and the CI was calculated (see Materials and Methods). Isobologram showing CI ≤ 1 indicates synergy.
a greater extent than did either drug alone (Fig. 1A and B). To determine whether the cytotoxic effect of the drug combination was synergistic or additive, we performed an analysis using the median effect method. HCT116 and HT-29 cell lines were treated with ABT-737 or CPT-11 for 48 h and their IC$_{50}$ values were calculated (Fig. 1C and D). The cell lines were treated with different concentrations of ABT-737 and CPT-11 at a fixed ratio (1:1) and cell viability was determined. The combination index (CI) was then calculated per the method of Chou and Talalay (22). As shown in an isobologram, the CI values in HCT116 cells were <1, indicating a synergistic interaction (Fig. 1C). In HT-29 cells, calculation of the CI revealed that the combination of ABT-737 and CPT-11 was additive (Fig. 1D).

We analyzed and quantified the apoptotic effect of ABT-737 or CPT-11 alone and in combination by Annexin V staining. ABT-737 plus CPT-11 induced apoptosis to a greater extent than did either drug alone in both cell lines (Fig. 2A-C). ABT-737 induced apoptosis to a greater extent in HCT116 versus HT-29 cells, which may be explained by lower levels of endogenous Mcl-1 and higher levels of Bcl-2 in HCT116 cells (Fig. 2D). Furthermore, the drug combination produced a 1.7-fold increase in cell death compared with ABT-737 (5 µmol/L) alone in HT-29 cells versus a 3.5-fold increase in HCT116 cells.

**ABT-737 and CPT-11 cooperatively enhance apoptotic signaling.** Exposure of HCT116 and HT-29 cell lines to the combination of ABT-737 plus CPT-11 resulted in enhanced activation of caspase-8, caspase-9, and caspase-3 and cleavage of Bid and poly(ADP-ribose) polymerase (PARP) compared with treatment with either drug alone (Fig. 3A). To determine whether drug-induced caspase-8 activation is due to a feedback amplification loop mediated by caspase-3 (23), we used a caspase-3 inhibitor (z-DEVD-FMK). z-DEVD-FMK was shown to attenuate caspase-8 cleavage by ABT-737 and its combination with CPT-11 in HCT116 cells (Fig. 3B), consistent with a...
feedback amplification loop mediated by caspase-3. To confirm that cell death occurred by an apoptotic mechanism, Bax knockout HCT116 cells were used. Treatment of these cells with ABT-737, CPT-11, or their combination failed to activate caspases or to cleave PARP (Fig. 3C), and their cytotoxic effects were completely abrogated (Fig. 3D).

Given that SN-38 is the active metabolite of CPT-11, we evaluated the effects of the combination of SN-38 and ABT-737 on caspase activation and cytotoxicity. SN-38 was used at nanomolar dosages because it has been shown to be ~1,000-fold more potent than CPT-11 (15, 24, 25). The combination with SN-38 and ABT-737 enhanced caspase cleavage (Fig. 4A) and cooperatively reduced cell viability (Fig. 4B) to a greater extent than did either drug alone in HCT116 cells. We determined the IC_{50} value for SN-38 (53.9 nmol/L) and evaluated the combination of SN-38 and ABT-737 at fixed ratios. Calculation of the CI of SN-38 and ABT-737 yielded CI < 1, indicating a synergistic interaction, as shown in an isobologram (Fig. 4C). We also measured apoptosis induction by SN-38, ABT-737, or their combination by Annexin V staining. The drug combination induced apoptosis to a greater extent than did either drug alone (Fig. 4D).

**ABT-737 displaces Bim and Bak from its complex with Bcl-xL/Bcl-2 or Bcl-xL.** We studied the mechanism by which ABT-737...
can potentiate CPT-11-induced apoptosis. As a BH3 mimic, ABT-737 binds to and neutralizes Bcl-2/Bcl-xL and can thereby disrupt important protein-protein interactions. Bim can bind to all prosurvival Bcl-2 proteins and is therefore a potent proapoptotic molecule (3). We determined the effect of ABT-737 treatment on the interaction between Bim and Bcl-2 proteins by immunoprecipitation of Bim and then probing for Bcl-xL, Bcl-2, and Mcl-1. ABT-737 treatment was shown to

![Image of immunoblotting results showing interactions between Bim and Bcl-2 proteins.](image)

**Fig. 4.** A, HCT116 cells were incubated with ABT-737 (10 μmol/L), SN-38 (10 nmol/L), or their combination for 24 h. DMSO was used as a vehicle control. The effects of drug treatment on caspase cleavage and Bid were analyzed by immunoblotting. B, cytotoxic effects of ABT-737 and/or SN-38 treatment for 48 h at indicated doses were determined using the MTS assay. Mean ± SD. Inset, treatment of HCT116 cells with SN-38 (10 nmol/L) was shown to induce Noxa expression. C, HCT116 cells were treated with increasing concentrations of SN-38 for 48 h and the cell viability was determined using the MTS assay. The IC50 value for SN-38 was calculated. Cells were treated with ABT-737 and SN-38 at a fixed ratio (500:1) and the CI was calculated (see Materials and Methods). An isobologram showing CI < 1 indicates synergy. D, apoptosis was determined using Annexin V and propidium iodide staining in HCT116 cells treated with vehicle (DMSO), SN-38, ABT-737, or their combination for 24 h at the indicated doses by FACS analysis.
Whole-cell lysates (vehicle DMSO) for 24 h and immunoprecipitation was done for Bim proteins. Whole-cell lysates were then probed for Bak or Bcl-xL proteins. ABT-737 also displaced Bim from its complex with Bcl-xL in Bak from Bcl-xL in determining the lethality of ABT-737 (26). We also observed that ABT-737 can induce Mcl-1 expression and Bim/HT-29 cells that lack endogenous Bcl-2 (Fig. 5B). We also observed that ABT-737 can induce Mcl-1 expression and Bim/ Mcl-1 complexes in both cell lines (Fig. 5A and B).

Recent studies have shown the importance of untethering Bak from Bcl-xL in determining the lethality of ABT-737 (26). Accordingly, we studied the effect of ABT-737 on the interaction between Bak and Bcl-xL in HCT116 and HT-29 cells. By immunoprecipitation of Bak and probing for Bcl-xL, we found that ABT-737 can disrupt the binding of Bak to Bcl-xL in both cell lines (Fig. 5C and D). Taken together, our data show that ABT-737 can activate Bak and unsequester Bim, which function in cellular commitment to apoptosis.

**CPT-11 up-regulates Noxa to sequester Mcl-1 and to disrupt Mcl-1/Bak complexes.** Noxa is a BH3-only protein whose expression can be induced by p53-dependent or p53-independent apoptotic stimuli (27). In HCT116 (wild-type p53) but not HT-29 (mutated p53) cells, CPT-11 treatment markedly induced expression of Noxa and also up-regulated Bak compared with vehicle-only treated cells (Fig. 6A). We also found that SN-38 significantly up-regulated Noxa expression in HCT116 cells (Fig. 4B). Given the induction of Noxa by CPT-11 in HCT116 cells, we determined the effect of drug treatment on the interaction between Noxa and its high-affinity partner Mcl-1 that result in Noxa/Mcl-1 complexes (3, 19). To address this issue, we immunoprecipitated Mcl-1 and probed for Noxa in HCT116 cells treated with CPT-11 or vehicle. CPT-11 treatment was shown to enhance the interaction of Mcl-1 and Noxa (Fig. 6B). It has been proposed that up-regulation of Noxa can activate Bak by displacing it from Mcl-1 (26). Consistent with this model, treatment with CPT-11 was shown to release Bak from its interaction with Mcl-1 (Fig. 6B).

Taken together, induction of Noxa by CPT-11 can sequester Mcl-1 and also release Bak from Mcl-1, which may contribute to the enhanced apoptotic effect of the CPT-11 and ABT-737 combination.

**Knockdown of Noxa attenuates the cytotoxicity of CPT-11 plus ABT-737.** To further show the importance of Noxa up-regulation as a potential mechanism for the synergistic cytotoxic effect of CPT-11 and ABT-737, we generated Noxa knockdown HCT116 cells using lentiviral-delivered shRNA. Knockdown of Noxa was found to significantly reduce sensitivity to CPT-11 plus ABT-737 as shown using two shRNA constructs (Fig. 6C; Supplementary Fig. S1). Furthermore, Noxa knockdown blocked the activation of caspase-3 induced by the drug combination (Fig. 6C). Because CPT-11 did not induce Noxa in HT-29 cells, we generated Noxa knockdown HT-29 cells. Knockdown of Noxa had no effect on the sensitivity of HT-29 cells to the combination of ABT-737 and CPT-11, although a slight protection was seen at an ABT-737 dose of 10 μmol/L (Fig. 6D). At a dose of 10 μmol/L, ABT-737 was shown to weakly induce Noxa (Fig. 6D) together, these data show that Noxa is a key regulator of apoptotic susceptibility to this drug combination.

To show that induction of Noxa represents an important mechanism to increase apoptotic susceptibility, we used the proteasome inhibitor bortezomib that has been shown to induce Noxa in human myeloma cells (19), mantle-cell lymphoma cells (29), and non-small cell lung cancer cells (30). Treatment of HCT116 and HT-29 cell lines with bortezomib (20-60 nmol/L) was shown to markedly induce Noxa in both cell lines (Fig. 7A). Co-administration of ABT-737 and bortezomib was shown to enhance caspase cleavage (Fig. 7B) and to enhance cytotoxicity in both cell lines to a greater extent compared with either drug alone (Fig. 7C). Using Noxa knockdown HCT116 and HT-29 cells, we observed that Noxa shRNA protected cells from caspase-3 cleavage and cytotoxicity induced by bortezomib plus ABT-737 (Fig. 7D). We also found that CPT-11 can induce Noxa in another colon cancer cell line, RKO, which expresses high levels of Mcl-1. In these cells, ABT-737 was again shown to enhance CPT-11-mediated cytotoxicity and caspase cleavage (Supplementary Fig. S2). These data suggest that our findings can be generalized to other colon cancer cell lines.

**Discussion**

ABT-737 is a potent small-molecule antagonist of prosurvival Bcl-2 proteins (8). Although sparse data exist as to the activity of ABT-737 in solid tumor cell lines, this drug has been shown to lower the apoptotic threshold for certain chemotherapeutic agents (8, 12). Colorectal cancers display intrinsic apoptosis resistance related, in part, to overexpression of prosurvival Bcl-2 proteins (2). Therefore, we determined whether the combination of ABT-737 and CPT-11 exerts an enhanced apoptotic effect...
Fig. 6. CPT-11 up-regulates Noxa to sequester Mcl-1 and to disrupt Mcl-1/Bak complexes. Knockdown of Noxa attenuates the cytotoxicity of CPT-11 plus ABT-737. The effect of CPT-11 treatment on the expression of BH3-only and proapoptotic proteins was determined. A, HCT116 and HT-29 cells were incubated with CPT-11 (10 μmol/L) for 48 h and whole-cell lysates were subjected to immunoblot analysis for the expression of indicated proteins. B, HCT116 cells were treated with vehicle (DMSO) or CPT-11 (10 μmol/L) for 24 h and immunoprecipitation was done for Mcl-1 proteins. Whole-cell lysates from beads and eluted proteins from beads were separated by SDS-PAGE and then probed for Mcl-1, Bim, Noxa, or Bak proteins. C and D, knockdown of Noxa was achieved using lentiviral-delivered shRNA in HCT116 (C, left) or HT-29 (D, left) cells and the level of Noxa expression was determined in untreated (vehicle) and drug-treated cells by immunoblotting. HCT116 cells (C, left) were treated with vehicle (DMSO) or the combination of ABT-737 and CPT-11 at the indicated doses for 24 h and caspase-3 cleavage was analyzed in whole-cell lysates subjected to immunoblotting. β-Tubulin served as a control for protein loading. We then determined the effect of Noxa knockdown versus control shRNA on cell viability in drug-treated HCT116 (C, right) and HT-29 (D, right) cell lines. Cell viability was determined using two Noxa shRNA constructs in HCT116 cells, and similar results for the Noxa and Noxa 3 (Supplementary Fig. S1) constructs were found. Cells were incubated with ABT-737 and/or CPT-11 for 48 h at the indicated doses and cell viability was determined using the MTS assay. Experiments were conducted in triplicate. Mean ± SD. *, P < 0.005; **, P < 0.0005.
Fig. 7. Bortezomib up-regulates Noxa to cooperatively enhance the effects of ABT-737 on cytotoxicity and apoptosis. A. HCT116 and HT-29 cells were treated with bortezomib (20-60 nmol/L) and Noxa expression was analyzed by immunoblotting. Cells were treated with bortezomib (20 nmol/L) or vehicle (DMSO) for 24 h and Bim, Puma, and Noxa expression was determined in whole-cell lysates by immunoblot analysis. B. HCT116 and HT-29 cells were incubated with vehicle (DMSO), ABT-737 (10 μmol/L), and/or bortezomib (20 nmol/L) for 24 h and their effects on caspase cleavage were analyzed in whole-cell lysates subjected to immunoblotting. β-Tubulin served as a control for protein loading. C. Cytotoxic effects of ABT-737 and/or bortezomib treatment for 48 h at indicated doses were determined in HCT116 and HT-29 cells using the MTS assay. Experiments were conducted in triplicate. Mean ± SD. D. Knockdown of Noxa was achieved using lentiviral-delivered shRNA in HCT116 (left) and HT-29 (right) cells. Cells were incubated with ABT-737 (10 μmol/L) and bortezomib (20 nmol/L) or vehicle (DMSO) for 24 h. Noxa expression and caspase-3 cleavage were then analyzed by immunoblotting. β-Tubulin served as a control for protein loading. The effects of ABT-737 and/or bortezomib treatment (48 h) at indicated doses on cell viability were determined using the MTS assay in the presence of Noxa shRNA or control shRNA-transduced HCT116 (left) and HT-29 (right) cell lines. Experiments were conducted in triplicate. Mean ± SD. *, P < 0.05; **, P < 0.005.
in human colon cancer cells. We found that ABT-737 monotherapy induced a dose-dependent apoptosis in HCT116 and HT-29 colon cancer cells. Moreover, we show that coadministration of ABT-737 with CPT-11 results in a synergistic (HCT116) or additive (HT-29) cytotoxic effect that is caspase-dependent and requires Bax as shown using Bax knockout HCT116 cells where caspase cleavage and apoptosis induction were completely abrogated. We also show that nanomolar doses of SN-38, the active metabolite of CPT-11, in combination with ABT-737 exerts a synergistic cytotoxic effect that is due to apoptosis. Highly concordant results with SN-38 and CPT-11 support the biological relevance of our observations.

The ability of ABT-737 to enhance apoptosis induction was due to its ability to disrupt the interaction of Bcl-xL with Bak and to displace Bim from its sequestration by Bcl-xL or Bcl-2 in both cell lines. Recent studies have shown the importance of unsequestering Bak from Bcl-xL in determining the lethality of ABT-737 (26). We also found that CPT-11 releases Bak from Mcl-1; together, these data for ABT-737 and CPT-11 are consistent with the indirect activation model (3). Recently, we (17) reported that ABT-737 can release Bim from Bcl-2 or Bcl-xL and that Bim shRNA can attenuate the cytotoxic effects of ABT-737 and TRAIL in human pancreatic cancer cell lines. Potentially, the ABT-737-induced dissociation of Bim from Bcl-2 or Bcl-xL may contribute to Bak activation. In this regard, Bim may act directly on Bax/Bak as shown by the observation that Bim, but not a Puma BH3 peptide, was sufficient to induce oligomerization and activation of Bax and Bak to permeabilize the outer mitochondrial membrane (5). Further evidence for the direct activation model is that activation of Bax or Bak by Bim has been shown in thymocytes from Bim/Bax or Bim/Bak double-knockout mice (31). Bim is a potent inducer of apoptosis because Bim, Puma, and tBid can neutralize all prosurvival Bcl-2 proteins, whereas Bad and Noxa show selectivity (6).

The ability of ABT-737 to release Bim from Bcl-xL or Bcl-2 may enable its complex formation with Mcl-1, as shown in both cell lines, and this Bim:Mcl-1 complex may attenuate the apoptotic effect of ABT-737. Unsequestered Bim has been shown to stabilize Mcl-1 (32) and this may account for the increase in Mcl-1 expression seen in ABT-737-treated cells. We found that CPT-11 or SN-38 treatment up-regulated Noxa expression in HCT116 cells, consistent with the role of BH3-only proteins, which act as molecular sensors of cellular stress or damage (6, 26, 33). Furthermore, CPT-11 was shown to increase Noxa/Mcl-1 complexes and also to disrupt the interaction of Mcl-1 with Bak. This finding is consistent with the observation that upregulated Noxa promotes Bak activation by displacing it from Mcl-1 (26, 29). Noxa can bind specifically to Mcl-1 and A-1 but not to Bcl-2 or Bcl-xL (6). Higher Noxa levels have been observed in cell lines sensitive to ABT-737, and ectopic expression of Noxa in a resistant cell line increased its sensitivity to ABT-737 (12). The converse has been shown for Mcl-1 in that ABT-737 binds to Mcl-1 with low affinity; thus, Mcl-1 reduces responsiveness to ABT-737 (12, 13). To confirm the importance of Noxa in the synergistic cytotoxicity of CPT-11 plus ABT-737, suppression of Noxa by shRNA was shown to markedly attenuate the cytotoxic effect of this drug combination and blocked caspase-3 cleavage in HCT116 cells. In HT-29 cells where CPT-11 did not induce Noxa, knockdown of Noxa failed to confer resistance to CPT-11 plus ABT-737. To confirm the role of Noxa in sensitizing cells to apoptosis, we used bortezomib that is known to induce Noxa in myeloma cells (19) and did so in our colon cancer cell lines. The combination of bortezomib and ABT-737 enhanced apoptosis induction in HCT116 and HT-29 cells compared with either drug alone, and this effect was attenuated using Noxa shRNA constructs.

In summary, we show that ABT-737 and CPT-11 induce a cooperative cytotoxic effect against human colorectal carcinomas cell lines. ABT-737 antagonizes Bcl-2/Bcl-xL to release Bim and Bak. Although ABT-737 does not target Mcl-1 (10, 11), our data indicate that CPT-11-induced up-regulation of Noxa can sequester Mcl-1 and thereby disrupt Mcl-1/Bak complexes to enhance apoptotic susceptibility. Together, these findings suggest that Noxa induction can sensitize tumors expressing Mcl-1 to ABT-737 and thereby suggest a strategy to improve the therapeutic efficacy of ABT-737 against human colorectal cancers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


Induction of Noxa Sensitizes Human Colorectal Cancer Cells Expressing Mcl-1 to the Small-Molecule Bcl-2/Bcl-x\textsubscript{L} Inhibitor, ABT-737

Kenji Okumura, Shengbing Huang and Frank A. Sinicrope