Mcl-1 Phosphorylation Defines ABT-737 Resistance That Can Be Overcome by Increased NOXA Expression in Leukemic B cells

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Abstract

ABT-737 is a small molecule Bcl-2 homology (BH)-3 domain mimetic that binds to the Bcl-2 family proteins Bcl-2 and Bcl-xL and is currently under investigation in the clinic. In this study, we investigated potential mechanisms of resistance to ABT-737 in leukemia cell lines. Compared with parental cells, cells that have developed acquired resistance to ABT-737 showed increased expression of Mcl-1 in addition to posttranslational modifications that facilitated both Mcl-1 stabilization and its interaction with the BH3-only protein Bim. To sensitize resistant cells, Mcl-1 was targeted by two pan–Bcl-2 family inhibitors, obatoclax and gossypol. Although gossypol was effective only in resistant cells, obatoclax induced cell death in both parental and ABT-737-resistant cells. NOXA levels were increased substantially by treatment with gossypol and its expression was critical for the gossypol response. Mechanistically, the newly generated NOXA interacted with Mcl-1 and displaced Bim from the Mcl-1/Bim complex, freeing Bim to trigger the mitochondrial apoptotic pathway. Together, our findings indicate that NOXA and Mcl-1 are critical determinants for gossypol-mediated cell death in ABT-737–resistant cells. These data therefore reveal novel insights into mechanisms of acquired resistance to ABT-737. Cancer Res; 72(12); 3069–79. ©2012 AACR.

Introduction

The interplay between Bcl-2 family members is essential for controlling the mitochondrial cell death pathway and thereby the survival of most cells, including those of hematopoietic origin. On the basis of their Bcl-2 homology (BH) domains, the Bcl-2 proteins have been grouped in 3 classes: antiapoptotic (containing the BH1-BH4 domains), proapoptotic, containing the BH1-BH3 domains, and those with the BH3 domain-only (1, 2). The members of this group regulate mitochondrial outer membrane permeabilization (MOMP), monitoring release of cytochrome c and activating downstream effector caspases (3). The imbalance in expression of these partners has been implicated in development of various tumor types and resistance to chemotherapeutic regimens (1). This often results from high-level expression of antiapoptotic members, such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1 that prevent cell death by sequestering BH3-only proteins, such as Bim, PUMA, and NOXA, and regulate activation of the proapoptotic proteins Bax and Bak. In most of these cases, upregulation and binding of significant amounts of antiapoptotic proteins to activator proteins keeps these cells alive (1, 2, 4, 5).

ABT-737 is a small molecule inhibitor that is effective against certain Bcl-2 family members. It has a strong affinity for Bcl-2, Bcl-xL, and Bcl-w that are bound to Bim (6) by releasing Bim from antiapoptotic Bcl-2 partners, thereby initiating MOMP. The oral derivative of ABT-737, navitoclax (ABT-263) is currently under investigation in several clinical trials in lymphoid malignancies, such as chronic lymphocytic leukemia (CLL), and tumors, such as small cell lung cancer (7–10). Importantly, ABT-737–mediated cell death is Bax/Bak dependent as Bax/Bak double knockout mouse fibroblasts are resistant to this treatment (11). However, it is expected that even for the most effective chemotherapeutics, acquired resistance remains a serious clinical problem; hence compounds that overcome drug resistance are of special interest in cancer therapy (7, 12–15). Studies with solution competition assays have shown that ABT-737 has very weak affinity for Mcl-1 (16). Various in vitro and in vivo studies have shown that sensitivity to ABT-737 is decreased in cells expressing elevated levels of Mcl-1 (5). Moreover, cells initially sensitive to ABT-737 become resistant by upregulating Mcl-1 levels (7).

To investigate the probable mechanisms of resistance to ABT-737, resistant cell lines were generated from pre–B tumor cells that developed increased levels of Mcl-1 protein that was also posttranslationally modified. These Mcl-1–dependent ABT-737–resistant cells (ABT-R) were exclusively sensitive to the pan–Bcl-2 inhibitor gossypol, but not obatoclax.
Knockdown of Mcl-1 or NOXA overcame gossypol sensitivity of ABT-R cells. Gossypol-induced, NOXA-dependent cell death led to release of Bim from Mcl-1 in ABT-R cells. These studies reveal novel insights into regulation and role of Mcl-1 in response to ABT-737 and provide mechanistic approaches for overcoming the acquired resistance to ABT-737 in leukemic cells.

Materials and Methods

Cell lines and reagents
Human B-cell acute lymphoblastic leukemia cell lines Nalm-6 and Reh were obtained from American Type Culture Collection. These pre-B cells express CD19 and CD127 surface markers with rearranged immunoglobulin heavy chains. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, Antibiotic–Antimycotic (Invitrogen). ABT-R cells were cultured in 5% FBS. Cell lines were routinely verified for growth rates, morphologic characteristics, and response to stimuli using Trypan blue exclusion or Annexin V/propidium iodide staining. Cell lines were periodically tested to be Mycoplasma free and their passage number did not exceed 20. ABT-737 was provided by Abbott Laboratories. Gossypol, actinomycin D, and cycloheximide were from Sigma-Aldrich and obatoclax from Selleck Chemicals.

Generation of ABT-737–resistant cell lines
Nalm-6 and Reh cells were cultured in increasing concentrations of ABT-737 administered intermittently, with the drug being washed off to allow cells to recover. Gradually, the ABT-737 concentration was increased until cells remained viable when ABT-737 concentrations double to that of their IC50 value was administered continuously. Cells were treated with verapamil (Sigma-Aldrich) to exclude the possibility of acquiring resistance due to increase in expression of drug efflux pumps (7, 17). The ABT-R cells were routinely monitored for resistance to ABT-737: they were cultured without drug for 72 hours before carrying out experiments.

Flow cytometry
Cell death was measured by phosphatidylserine externalization (5), by staining with fluorescein-conjugated Annexin V (BD Biosciences) and propidium iodide, and analyzed on a BD FACS Calibur flow cytometer. The raw data obtained was analyzed by CellQuest Version 5.2.1 software. The results were normalized to survival of control cells that have been treated with dimethyl sulfoxide (DMSO) or ethanol.

Immunoblotting and immunoprecipitation
Protein lysates were prepared with 1% NP-40 lysis buffer (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L EDTA; 1% NP-40) containing protease and phosphates inhibitors for 1 hour. Protein lysates were incubated with primary antibody overnight at 4°C followed by 1 hour of incubation of protein A agarose beads (eBioscience; Calbiochem) at 4°C. Immunoprecipitates were washed 3 times with CHAPS and eluted with loading buffer. The coimmunoprecipitated proteins were resolved by SDS-PAGE. For Bax activation, cells were lysed with 1% CHAPS lysis buffer for 1 hour at 4°C and active Bax was immunoprecipitated with 6A7 (BD Biosciences) and probed with N20 (Santa Cruz Biotechnology) antibodies. Primary antibodies used were for Mcl-1, Bim (BD-Biosciences), NOXA (Enzo Life Sciences), Bcl-2, Bcl-xl. (Santa Cruz Biotechnology), PUMA (ProSci Corporation), USP-9X (Abnova), PARP1 (Cell Signaling), Mcl-1 (5-64; ref. 18; provided by Dr. Gregory J. Gores, Mayo Clinic, Rochester, MN), Mcl-1 (Thr-163) (Cell Signaling), phospho-Erk (Cell Signaling), and β-actin (Sigma).

RNA isolation and real-time quantitative PCR
RNA isolated from parental and ABT-R cells after ABT-737 treatment (500–1,000 mmol/L) for 4 to 24 hours was examined by quantitative reverse transcriptase PCR (qRT-PCR), using primers for Mcl-1 and NOXA, and normalized for β-actin as described (5). Similar experiments were carried out with ABT-R cells followed by gossypol (10 mmol/L) at 12 hours. The half-life of Mcl-1 mRNA was determined following actinomycin D treatment and qRT-PCR analysis.

Genetic manipulation of Mcl-1 and NOXA
NOXA knockdown was achieved in ABT-R Nalm-6 and Reh cells by short hairpin RNA (shRNA) delivered by lentivirus transduction (5). Single cell clones with stable expression of shNOXA (Santa Cruz; sc-37305-V), shMcl-1 (Sigma-Aldrich; NM_021960.3-3125s1c1, NM_021960.3-1001s1c1) and scrambl shRNA (control; sc-108080) were selected with puromycin (Sigma-Aldrich). NOXA shRNA lentiviral particles were obtained as a pool of concentrated, transduction-ready viral particles containing 3 target-specific constructs that encode 19 to 25 nt (plus hairpin). Mcl-1 shRNA lentiviral particles were prepared according to manufacturer’s protocol.

Statistical analysis
Statistical comparisons between groups were conducted by 2-way ANOVA using Prism software. SD was calculated from experiments conducted in triplicates and indicated as error bars. All experiments were repeated 3 times independently.

Results
Acquired resistance of B-cells to ABT-737 after prolonged exposure
ABT-737 is a BH3-mimetic that binds only to certain Bcl-2 family proteins, such as Bcl-2, Bcl-xl, and Bcl-w, but not Mcl-1. In our previous studies, we have shown that patients with increased Mcl-1 levels do not respond to ABT-737 and thereby become resistant to this drug (5). Hence, to investigate the mechanism responsible for resistance, we generated ABT-737–
resistant (ABT-R) cell lines from initially sensitive pre-B Nalm-6 and Reh cells. These cell lines were selected for their sensitivity to ABT-737, with IC_{50} values of approximately 1,000 and 500 nm, respectively. The Nalm-6 ABT-R and Reh ABT-R cells generated could tolerate continuous exposure to ABT-737 at a concentration double to that of their IC_{50} value (Fig. 1A). It has been known that ABT-737 triggers Bax/Bak-mediated apoptosis (11). ABT-737 treatment led to Bax activation, as indicated by 6A7 antibody reactivity in Nalm-6 and Reh cells, but not in their resistant derivatives (Fig. 1B). These results are in support of how resistant cells could evade Bax-mediated apoptosis caused by ABT-737. Further examination of Bcl-2 family proteins by immunoblotting in parental and ABT-R cells indicated increased Mcl-1 levels in ABT-R cells (Fig. 1C). Bfl-1 levels were below our detection limit. We could also observe decreased Bcl-2 and increased Bim levels in Reh ABT-R cells, which were comparable with those found in Nalm-6 ABT-R cells. PUMA and NOXA expression was not markedly altered (Fig. 1C). Mcl-1 protein levels increased in parental but not in ABT-R cells following acute ABT-737 treatment (Fig. 1D).

Figure 1. Acquired resistance development in leukemic cells. A, parental and ABT-737-resistant (ABT-R) Nalm-6 and Reh cells were treated with ABT-737 for 24 hours. Cell viability is shown as a percentage of Annexin V–FITC/propidium iodide negative, relative to control cells treated with DMSO, as determined by flow cytometric analyses. SD is indicated as error bars (n = 3). B, cells were treated with ABT-737 for 24 hours with 1,000 nmol/L (Nalm-6) or 500 nmol/L (Reh) ABT-737, lysed with 1% CHAPS lysis buffer, and 1 mg protein was immunoprecipitated for 6A7-specific Bax and immunoblotted for total Bax. C, whole-cell lysates were analyzed by immunoblotting for expression of Bcl-2 family proteins and β-actin by immunoblotting with the indicated primary antibodies. D, parental and ABT-R Nalm-6 cells were treated with ABT-737 (1,000 nmol/L for 1–3 hours) and expressions of Bim, USP 9X, Mcl-1, PARP1, and β-actin was determined by immunoblotting. The results in B to D are representative of 3 independent experiments. IP, immunoprecipitation.
In addition, there was no further change of Mcl-1 levels in ABT-R cells following 18 hours of ABT-737 exposure (Supplementary Fig. S1C). Parental cells were sensitive to ABT-737 and within 3 hours, they were committed to cell death, as indicated by cleavage of PARP1. Increased Mcl-1 levels were attributed to increased deubiquitination as a result of increased levels of the USP9X deubiquitinating enzyme (19); however, USP9X levels (Fig. 1D) and its association with Mcl-1 (data not shown) did not change in ABT-R cells. Our results showed that ABT-737–resistant cells could evade Bax-mediated cell death and develop resistance by elevating Mcl-1 levels.

**ABT-737 resistance is associated with increased Mcl-1 protein stability**

Previous studies of acquired ABT-737 resistance reported that increased expression levels of Mcl-1 in ABT-R cells are a result of increased mRNA levels (7). However, there was no difference in constitutive Mcl-1 and NOXA mRNA levels in resistant compared with parental Nalm-6 and Reh cells, as determined by qRT-PCR (Supplementary Fig. S1A). Similar results were found for Bim-1 (data not shown). Most importantly, there was no change in Mcl-1 mRNA levels following chronic ABT-737 treatment in Nalm-6 (1 μmol/L) and Reh (500 nmol/L) parental and ABT-R cells (Supplementary Fig. S1B). These results indicated that Mcl-1 was not transcriptionally upregulated; however, this does not preclude the possibility of a reduced turnover of the Mcl-1 transcript in ABT-737–resistant cells. ABT-R Nalm-6 and Reh cells examined up to 2 hours following treatment with actinomycin D, a known transcriptional inhibitor, indicated that the half-life of Mcl-1 mRNA was not altered (data not shown). Because the Mcl-1 protein has a very short half-life, Mcl-1 protein stability was further evaluated. Immunoblotting following 30 to 90 minutes of cycloheximide treatment revealed that the half-life of Mcl-1 protein was increased considerably, to 60 to 90 minutes in ABT-R compared with 15 to 20 minutes in parental cells (Fig. 2A–C). These results indicated that increased Mcl-1 levels of ABT-R cells were due to its stabilization at the protein and not at mRNA level.

**Increased Mcl-1 levels sequester Bim following its displacement from Bcl-2 and Bcl-xL complexes in ABT-R cells**

It has been reported that increased Mcl-1 levels in ABT-R cells associate with Bim after its competitive dissociation from Bcl-2/Bcl-xL complexes by ABT-737. Importantly, displacement of Bim from Bcl-2/Bcl-xL complexes by ABT-737 in parental, sensitive cells was shown to be a critical event for
committing cells to death (7). Indeed, our coimmunoprecipitation analyses indicated that association of Mcl-1 with Bim in ABT-R cells was more pronounced compared with those in parental cells. On the other hand, there was more Bim present that was not bound to Mcl-1 in parental cells, instead was found in the supernatant following immunodepletion of Mcl-1 (Fig. 3A, right). These results were confirmed by reciprocal immunoprecipitation–immunoblot analyses with Mcl-1 and Bim, respectively (Fig. 3A, left).

We next investigated the interaction of Bim with Bcl-2/Bcl-xL in parental and ABT-R Nalm-6 cells. There was less association of Bim with Bcl-2/Bcl-xL in ABT-R compared with parental Nalm-6 cells, which was in contrast to binding of Bim to Mcl-1 (Fig. 3A, left). Taken altogether, these data suggested that Bim was dissociated from Bcl-2/Bcl-xL complexes by competitive displacement by ABT-737. Moreover, this displaced Bim was bound to Mcl-1 in resistant but not in parental cells, even when ABT-737 was absent. In contrast, in parental cells, even though Mcl-1 levels increased following acute ABT-737 treatment, Mcl-1 could not bind to Bim (Fig. 3A and B). Similar observations were made in Reh ABT-R cells (data not shown).

We next explored the mechanism responsible for the differences in the ability of Mcl-1 to bind Bim in ABT-R compared with parental cells. It has been shown that Mcl-1 Ser-64 phosphorylation is associated with enhanced binding to proapoptotic proteins, such as Bim, NOXA, and Bak (18). Interestingly, Mcl-1 was phosphorylated on Ser-64 in ABT-R Reh and Nalm-6, but not in parental cells (Fig. 3C). In contrast, although Mcl-1 levels increased, nevertheless Mcl-1 Ser-64 phosphorylation was not detected in Nalm-6 following 6 hours of acute treatment with ABT-737 (Fig. 3C, left). These findings suggested that Mcl-1, the levels of which increased following ABT-737 treatment in parental cells, failed to associate with Bim due to lack of Ser-64 Mcl-1 phosphorylation. It has been reported that Mcl-1 phosphorylation also regulates Mcl-1 protein stability, with Thr-163 Mcl-1 phosphorylation by extracellular signal–regulated kinases (Erk) extending its half-life (20). Indeed, Mcl-1 was phosphorylated on Thr-163 in ABT-R Nalm-6 and Reh, but not in parental cells (Fig. 3D, left). Erk was also activated, as indicated by p-Erk expression in ABT-R but not the parental cells (Fig. 3D, right). These results indicated that phosphorylation of Mcl-1 at specific residues has developed during acquired resistance to ABT-737 treatment. These Mcl-1 posttranslational modifications facilitate its interaction with Bim, leading to increased Mcl-1 protein stability in ABT-R cells, as previously suggested (21, 22).

**Gossypol sensitizes ABT-737–resistant cells by upregulating NOXA**

To overcome Mcl-1–dependent acquired resistance, parental and resistant cells were treated with increasing concentrations of gossypol. Annexin V fluorescein isothiocyanate (FITC)–propidium iodide staining (Supplementary Fig. S2A) and trypan blue exclusion (Fig. 4A) indicated that ABT-R cells were exquisitely sensitive to gossypol treatment compared with parental cells. In addition, cyclin E cleavage, another marker of apoptosis (23, 24), was generated in ABT-R cells (data not shown). Time course experiments with gossypol showed maximum cell death at 24 hours. To study the mechanism of its action and avoid consequences of cell death, the 18-hour time point and a 10 μmol/L of gossypol dose were chosen as the minimum concentration at which ABT-R but not parental cells were most sensitive (all other experiments used gossypol at 10 μmol/L for 18 hours, unless otherwise stated). Mcl-1 protein stability was not altered substantially following gossypol treatment in ABT-R cells (data not shown). The levels of antia apoptotic and proapoptotic proteins were next examined in ABT-R Nalm-6 (left) and Reh cells (right) following a 6- to 18-hour exposure to gossypol (Fig. 4B). Interestingly, a low molecular weight Mcl-1 species was present, most likely an alternative splice variant of Mcl-1 (Mcl-1s), which has been reported to function as a proapoptotic molecule (25). The expression of NOXA increased greatly in a time-dependent manner (Fig. 4B). Bcl-xL and Bcl-2 levels were downregulated following treatment in Reh ABT-R cells (Fig. 4B, right); however, PUMA levels did not change significantly (data not shown). There was a modest increase in Mcl-1 levels in Nalm-6 cells following gossypol treatment, and those of NOXA also increased less compared with ABT-R cells (Supplementary Fig. S2B). When NOXA and Mcl-1 mRNA levels were examined at 12 hours following gossypol treatment, there was a 5- to 6-fold increase of Mcl-1 mRNA and a robust 20- to 25-fold induction of NOXA levels in ABT-R cells (Fig. 4C). These findings indicated that upregulation of Mcl-1 and NOXA following gossypol treatment was at mRNA level.

It has been reported that obatoclax (GX15-070), similar to gossypol, targets Mcl-1 (26) and is predicted to overcome Mcl-1–dependent ABT-737 resistance (27). Indeed, Nalm-6 ABT-R cells were sensitive to obatoclax; however, Nalm-6 cells were equally sensitive (IC50: 10 μmol/L). Reh parental cells (IC50: 5 μmol/L) were also more sensitive to obatoclax compared with ABT-R cells, as measured by trypsin blue exclusion (Supplementary Fig. S3A). Mcl-1 protein levels were downregulated following obatoclax treatment. NOXA levels also increased, however these changes did not correlate with cell death (Supplementary Fig. S3B). Hence, gossypol was used for further studies, as it selectively sensitizes ABT-R cells.

**NOXA expression mediates gossypol-induced cell death**

To determine whether NOXA induction and cell death are a consequence of caspase activation, ABT-R Nalm-6 and Reh cells were treated with a pan-caspase inhibitor before gossypol treatment. zVAD-fmk addition prevented caspase activation, but NOXA induction and generation of Mcl-1(s; Fig. 5A). To directly show a critical role for NOXA for the gossypol response, NOXA was depleted by shRNA in ABT-R Nalm-6 and Reh cells, under conditions in which Mcl-1 and Bim levels were unaltered. Remarkably, NOXA knockdown (Fig. 5C) prevented significantly gossypol-induced cell death of ABT-R Nalm-6 (P < 0.003) and Reh cells (P = 0.008; Fig. 5B), thus showing the critical role played by NOXA in the cytotoxic gossypol response. In addition, shRNA-mediated Mcl-1 knockdown in ABT-R cells (2 independent clones) restored resistance to gossypol treatment as measured by trypan blue exclusion (Nalm-6 ABT-R, P = 0.001; Reh ABT-R, P = 0.007;
Figure 3. Increased Mcl-1 levels are associated with Bim in ABT-R cells. A, Bim was immunoprecipitated and immunoblot analyses were carried out for Mcl-1, Bcl-2, Bcl-xL, and Bim (left). Reciprocal immunoprecipitation–Western blot analysis was carried out for Mcl-1 followed by immunoblotting for Bim to determine Mcl-1–bound and free Bim (right). B, Bim was immunoprecipitated from cells treated with ABT-737 (1,000 nmol/L) for 3 hours and immunoblotted for Mcl-1 and Bim (left) to determine the proportion of Mcl-1–bound and Mcl-1–free Bim (right). C, Mcl-1 was immunoprecipitated from ABT-737–treated Nalm-6 or untreated ABT-R cells followed by immunoblot analysis for Ser-64-Mcl-1, Mcl-1, and IgG (left). Similar analyses were carried out with parental and ABT-R Reh cells (right). D, Mcl-1 immunoprecipitated and analyzed by immunoblotting for Thr-163-Mcl-1 (left). Whole-cell lysates were analyzed by immunoblot for phospho-Erk expression and β-actin (right). The results in A to D are representative of 3 independent experiments. IP, immunoprecipitation.
Supplementary Fig. S2D) and PARP1 cleavage (data not shown). These data showed that gossypol is more effective in Mcl-1–dependent ABT-R cells.

**Association of NOXA with Mcl-1 leads to release of Bim following gossypol treatment**

Next, to address the mechanism of sensitization by gossypol, the Mcl-1/Bim association was examined. Bim immunoprecipitation revealed that Mcl-1 was no longer associated with Bim in Nalm-6 ABT-R cells following gossypol treatment, indicating that the Bim/Mcl-1 complex was disrupted (Fig. 6A, left). In contrast, Bcl-2 and Bcl-xL were still bound to Bim in these Nalm-6 and Reh ABT-R cells (Supplementary Fig. S2C). Moreover, Mcl-1 pull-down revealed that NOXA interaction with Mcl-1 facilitated Bim release from the Mcl-1/Bim complex in ABT-R cells (Fig. 6B). The released Bim, present in the supernatant (Supplementary Fig. S2E), most likely initiated gossypol-induced mitochondrial cell death. The Bim/Mcl-1 complex was also disrupted by NOXA in parental cells, but to a lesser extent, and with a slower kinetics compared with ABT-R cells (Fig. 6A). To determine the direct effect of NOXA on the Mcl-1/Bim association after gossypol treatment, NOXA levels were downregulated by shRNA. Interestingly, shRNA-mediated NOXA knockdown not only prevented cell death but also the release of Bim from Mcl-1 and Mcl-1(s) genesis in ABT-R Nalm-6 and Reh cells following gossypol treatment (Fig. 5B, 6C). These results indicated that the association of NOXA with Mcl-1 in ABT-R cells following gossypol treatment led to release of Bim that was critical for inducing mitochondrial cell death.

**Discussion**

Acquired resistance is a concern for chemotherapeutic treatments used for lymphoma, CLL, and other malignancies. Although there have been basic advances in understanding the mechanisms for this resistance, few efforts have been made to study the contribution of posttranslational regulation of proteins that leads to such resistance. The focus of our current studies was to understand the mechanism responsible for acquired ABT-737 resistance in leukemic cells and how to
overcome it selectively. Nuclear magnetic resonance studies have shown that ABT-737 binds to Bcl-2, Bcl-xl, and Bcl-w at sub-nmol/L concentrations, but not to Mcl-1. Hence, Mcl-1 levels can determine ABT-737 sensitivity (7). Moreover, inherent increased levels of these proteins can be a frequent cause of resistance. Various drugs, such as flavopiridol, R-roscovitine (Seliciclib), and PHA 767491 that downregulate Mcl-1 at mRNA level are being tested for sensitizing such tumor cells (7, 28). Recently, sorafenib (BAY43-9006; Nexavar) was shown to induce apoptosis by downregulation of Mcl-1 at translational rather than posttranslational level (29-32). However, due to lack of in vivo studies, except for ABT-737 and gossypol, most of the drugs acting as BH3 mimetic or Bcl-2 antagonists in clinical trials may have potential toxicities (12).

This study has examined the molecular mechanism for acute versus chronic response to ABT-737 in leukemic cells. Recent studies and our previous report indicate that increased levels of Mcl-1 accumulate in ABT-R cells, with Mcl-1 being bound to Bim significantly more in resistant than parental cells (5, 7). In our leukemic model of ABT-737 resistance, Mcl-1 was not upregulated at transcriptional level, which led us to explore its posttranslational regulation. Previous studies have shown that posttranslational modifications, such as phosphorylation of Mcl-1 at specific residues, are important for its binding to BH3-only proteins and its stability (18, 33). Here, we have shown that in ABT-R cells, phosphorylation of Mcl-1 at Ser-64 facilitates association of Bim with Mcl-1. Ser-64 Mcl-1 was not detected in parental cells, not even following acute treatment with ABT-737 and it is, rather, acquired during the course of resistance. Moreover, Mcl-1 was also phosphorylated on Thr-163 in ABT-737-resistant cells through Erk activation. Because Erk pathway was activated, we next studied its role on posttranslational regulation that could impact on Mcl-1 turnover. It has been shown that Erk phosphorylates Thr-163 on Mcl-1.
within its PEST region and stabilizes it (20). It has been suggested that Thr-163 is the priming phosphate that initiates Ser-159 phosphorylation of Mcl-1 through GSK3β leading to its ubiquitination and degradation (34). Clearly, phosphorylation at Thr-163 but not subsequent Ser-159 phosphorylation on Mcl-1 in ABT-R cells supports why Mcl-1 was more stable in ABT-R compared with parental cells. Mcl-1 is a short-lived protein and it is a target of E3 ubiquitin ligases that mediate its proteasomal mediated degradation. Mcl-1 is inaccessible to E3-ubiquitin ligases FBW7 and SCFβ-TrCP when it is bound to BH3-only proteins, such as Bim, as more association of Bim with Mcl-1 prevents their access to Mcl-1. As there is competitive binding between these E3-ubiquitin ligases and BH3-only proteins because they share the same C-terminal--binding

Figure 6. Interaction of NOXA with Mcl-1 triggers the release of Bim from Mcl-1 following gossypol treatment. A, Bim was immunoprecipitated from gossypol-treated cells followed by immunoblotting to determine Mcl-1 association (left). Immunoprecipitation for Mcl-1 was followed by immunoblotting for NOXA (right). B, association of NOXA and Bim with Mcl-1 was determined by immunoprecipitation–immunoblot analysis in gossypol-treated cells. C, Mcl-1 immunoprecipitates from cells following NOXA knockdown and gossypol treatment were analyzed by immunoblotting for NOXA, Bim, and Mcl-1. Data shown are representative of 3 independent experiments. D, model for ABT-737 resistance. ABT-737 does not target Mcl-1, which confers resistance by sequestering Bim displaced from Bcl-2. The Mcl-1/Bim association was enhanced by phosphorylation of Mcl-1, thereby preventing Bax activation. E, model for overcoming ABT-737 resistance by gossypol. Gossypol induces NOXA that binds to p-Mcl-1 and displaces Bim from the Mcl-1/Bim complex, leading to cell death that is augmented by Mcl-1s (splice variant) generation by gossypol treatment. IP, immunoprecipitation.
Bim is no longer released from the Mcl-1/Bim complex in ABT-R cells. Approaches to overcome resistance toward a single agent rely on targeting cells with another agent to exploit dependability of cells on the resistance determinant. Response to chronic exposure to ABT-737 leads to displacement of Bim from Bcl-2, Bcl-xL, and Bcl-w, which is then captured by another prosurvival protein, Mcl-1. In ABT-737–resistant cells levels of Mcl-1, its stability and affinity toward Bim is increased, making them Mcl-1 dependent. To target this induced Mcl-1 primed with Bim in ABT-R cells, 2 pan-Bcl-2 family antagonists were examined. Gossypol has a significant antitumor activity against lymphoma, head and neck, and prostate cancer through induction of NOXA and PUMA (35–37). Obatoclax is a small molecule that disrupts interaction between Mcl-1 and Bak and hence shows potential to overcome ABT-737 resistance (27). Although obatoclax was effective against ABT-R cells, it did not have specificity for ABT-737–resistant cells. In contrast, gossypol emerges from this study as a very potent compound against ABT-737–resistant leukemic cells that could target Mcl-1 indirectly by inducing NOXA. We have previously reported that primary cells from CLL patients with elevated Mcl-1 levels are less responsive to ABT-737 (5). Here we show that gossypol preferentially targets ABT-R with elevated Mcl-1, presumably, that is also phosphorylated Mcl-1 found in cells and causes cell death by inducing NOXA. shRNA-mediated downregulation of Mcl-1 in ABT-R cells decreased gossypol sensitivity, indicating that elevated levels of Mcl-1 are the cause of gossypol sensitivity in conjuction with NOXA induction following gossypol treatment. NOXA induction in our study is at the mRNA level. The resulting increased NOXA levels following gossypol treatment then associate with Mcl-1, leading to release of Bim from the Bim/Mcl-1 complex and thereby neutralize the antiapoptotic function of Mcl-1. Mcl-1–free Bim then initiates Bax/Bak activation (38, 39) and triggers the mitochondrial apoptotic pathway. A similar mechanism was reported to be responsible for apoptosis induced by bortezomib in multiple myeloma (31) and HeLa cells following ER stress and proteasome inhibition (40). NOXA was reported to have the highest binding affinity to Mcl-1 compared with other antiapoptotic Bcl-2 family members (41–45). Moreover, as following NOXA knockdown Bim is no longer released from the Mcl-1/Bim complex in ABT-R cells, hence NOXA specifically displaces Bim from Mcl-1 in these cells following gossypol treatment. Gossypol-induced cell death was clearly NOXA dependent as prevention of NOXA expression significantly prevented cell death. Generation of an alternative, proapoptotic splice variant of Mcl-1, Mcl-1(s) has also a significant contribution to gossypol-induced cell death. Mcl-1(s) was not generated by caspase-mediated proteolytic cleavage, as suggested earlier (30–32), as zVAD-fmk addition prevented caspase-3 activation but not the appearance of this faster migrating form of Mcl-1 corresponding to Mcl-1(s). Mcl-1 mRNA induction by gossypol facilitated generation of Mcl-1(s), which can also contribute to mitochondrial cell death pathway. Moreover, gossypol-induced cell death was NOXA dependent as prevention of NOXA expression significantly prevented cell death. In summary, gossypol-induced cell death is dependent on Mcl-1 expression and its phosphorylation in ABT-R cells that is targeted by NOXA induction and subsequent binding to Mcl-1, thereby displacing Bim, leading to cell death (Fig. 6D and E). Gossypol, a natural product, has been shown to be effective in inducing apoptosis as well as autophagy-mediated cell death in various cancer cell lines and xenograft tumor models as pan-Bcl-2 inhibitor (35, 36, 46). It is possible that apogossypol, its semisynthetic derivative with structural modifications that increases its affinity for antiapoptotic Bcl-2 proteins and reduces its toxic effects could be also effective in ABT-737–resistant patients, as both are under evaluation in phase I/II clinical trials for CLL and several other malignancies, such as prostate, lung, and brain tumors. These findings provide insights into the molecular mechanism of ABT-737 resistance and how it can be overcome with gossypol, which can be considered for clinical treatment of navitoclax-resistant leukemia patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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