

Mechanisms of Antileukemic Activity of the Novel Bcl-2 Homology Domain-3 Mimetic GX15-070 (Obatoclox)

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Abstract

In this study, we investigated the mechanism of apoptosis induction of obatoclox (GX15-070), a novel Bcl-2 homology domain-3 (BH3) mimetic, in acute myeloid leukemia (AML) cell lines and primary AML samples. Obatoclox inhibited cell growth of HL-60, U937, OCI-AML3, and KG-1 cell lines. Apoptosis induction contributed to the observed antiproliferative effects at concentrations of this agent that mirror its affinity for antiapoptotic Bcl-2 proteins. We show that obatoclox can promote the release of cytochrome *c* from isolated leukemia cell mitochondria and that apoptosis induced by this agent is preceded by the release of Bak from Mcl-1, liberation of Bim from both Bcl-2 and Mcl-1, and the formation of an active Bak/Bax complex. Notably, apoptosis was diminished, but not fully prevented, in the absence of Bak/Bax or Bim, suggesting that obatoclox has additional targets that contribute to its cytotoxicity. At growth inhibitory doses that did not induce apoptosis or decrease viability, obatoclox induced an S-G₂ cell-cycle block. Obatoclox induced apoptosis in AML CD34+ progenitor cells with an average IC₅₀ of 3.59 ± 1.23 μmol/L although clonogenicity was inhibited at concentrations of 75 to 100 nmol/L. Obatoclox synergized with the novel BH3 mimetic ABT-737 to induce apoptosis in OCI-AML3 cells and synergistically induced apoptosis in combination with AraC in leukemic cell lines and in primary AML samples. In conclusion, we show that obatoclox potently induces apoptosis and decreases leukemia cell proliferation and may be used in a novel therapeutic strategy for AML alone and in combination with other targeted agents and chemotherapeutics. [Cancer Res 2008;68(9):3413–20]

Introduction

Induction of apoptosis through the intrinsic apoptotic pathway is triggered by activation and oligomerization of the proapoptotic Bcl-2 family proteins Bax and Bak, which permeabilize the outer mitochondrial membrane to release apoptogenic factors, like cytochrome *c*, AIF, endoG, and omi/htra2 (1). For Bax and Bak to oligomerize, they must first be liberated from antiapoptotic Bcl-2

family proteins and endogenous proteins that contain a conserved dimerization motif termed Bcl-2 homology domain-3 (BH3), bind to antiapoptotic Bcl-2-family members, and promote the release of Bax and Bak (2). Notably, a recent report shows that antagonizing the antiapoptotic Bcl-2 proteins that sequester Bax and Bak is necessary and sufficient to induce apoptosis (3), explaining the spectacular single-agent activity reported for the novel BH3 mimetic ABT-737 in lung cancer xenografts (4). Albeit ABT-737 potently antagonizes most antiapoptotic Bcl-2 family proteins, it does not antagonize Mcl-1, and we have shown accordingly that in acute myeloid leukemia (AML) cells Mcl-1 confers complete resistance to ABT-737-induced apoptosis (4, 5). Therefore, it is of great interest to identify agents that can antagonize the antiapoptotic action of Mcl-1.

In this report, we investigate the activity of the novel BH3 mimetic obatoclox (GX15-070) in AML cell lines and primary samples. Obatoclox has been reported to similarly antagonize all antiapoptotic Bcl-2 family proteins (average IC₅₀, 3 μmol/L), including Mcl-1 (IC₅₀, 2.9 μmol/L) and Bfl-1 (IC₅₀, 5 μmol/L; ref. 6), and the clinical formulation of this agent is currently being evaluated in several phase I and phase II trials. We first sought to determine if apoptosis contributed to the antiproliferative effects of obatoclox and found that concentrations that antagonize Mcl-1 and Bcl-2, as evidenced by the release of Bak and Bim, induced apoptosis by activation of the intrinsic pathway. However, unlike observed for ABT-737, apoptosis induced by this agent was only partially dependent on Bak/Bax or Bim, suggesting that in cells treated with obatoclox, targets (other than Bcl-2 proteins) contribute to its cytotoxicity. Secondly, we identified that obatoclox can induce an S-G₂ cell cycle arrest that mediates its potent growth inhibitory effects, suggesting that in addition to antagonizing antiapoptotic Bcl-2 proteins, the cycloprodigiosin structure of this agent may have other targets. Lastly, we observed that obatoclox effectively induced apoptosis of primary AML samples and found that this was associated with release of Bim from Bcl-2. Our observations support the therapeutic use of obatoclox alone and in combination with AraC and ABT-737, and we propose that the liberation of Bim from Bcl-2 may serve as a biomarker of activity of this agent.

Materials and Methods

Reagents and antibodies. DMSO and trypan blue were purchased from Sigma Chemical Co. Annexin V APC was purchased from BD Biosciences. The cytochrome *c*, Bax, Mcl-1, and Bcl-2 antibody used for immunoprecipitation were purchased from BD Biosciences, the Bcl-2 antibody for immunoblotting from Dako, and the Bak antibody from Upstate. Bim and activated Bak antibodies were purchased from Calbiochem. The Mcl-1 antibody used for immunoprecipitation was purchased from Santa Cruz Biotechnology, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-1919

antibody from Chemicon International, and goat anti-mouse and goat anti-rabbit-horse radish peroxidase conjugate secondary antibodies from Bio-Rad.

Cell lines and primary AML samples. U937, HL-60, and KG1 cells were purchased from American Type Culture Collection. OCI-AML3 cells were kindly provided by Mark Minden (Ontario Cancer Institute); Bak knockout mouse embryo fibroblasts (MEF) by Anthony Letai (Dana-Farber Cancer Institute); and Bim knockout MEFs by Philippe Bouillet (Walter and Eliza Hall Institute of Medical Research).

Bone marrow or peripheral blood samples were obtained for *in vitro* studies from patients diagnosed with AML during routine diagnostic workup under informed consent in accordance with regulations and protocols approved by the Institutional Review Board Committee of The University of Texas M. D. Anderson Cancer Center. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical Co.) density-gradient centrifugation. Cells were either used for colony assays, as described below, or cultured in AIM-V medium (Life Technologies Laboratories) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products), 1 mmol/L L-glutamine (Life Technologies Laboratories), and 50 µg/mL penicillin/streptomycin (Life Technologies Laboratories).

Cell culture. U937, HL-60, KG1, and OCI-AML3 cells were cultured in RPMI 1640 (Mediatech, Inc.). MEF cells were cultured in DMEM (Mediatech, Inc.). All media were supplemented with 10% FBS, 1 mmol/L L-glutamine, and 50 µg/mL penicillin/streptomycin.

Leukemic cell lines and mononuclear cells from AML patients were cultured at a density of 3.0×10^5 cells/mL in medium supplemented with 10% FBS and treated with either obatoclast or vehicle (DMSO final concentration, 0.1%). Obatoclast was dissolved in DMSO to yield a stock of 10 mmol/L, which was diluted into the culture medium to the indicated concentrations. In all experiments, cells were treated in log-phase growth.

Viability assay. The number of viable cells was assessed using a Vi-CELL XR cell viability analyzer from Beckman Coulter at 72 h posttreatment.

Flow cytometric analysis of apoptosis. Apoptosis was determined by the flow cytometric detection of phosphatidylserine externalization using Annexin V APC (BD Biosciences). Briefly, cells were washed twice with binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl, and 5 mmol/L CaCl₂ (pH 7.4), all from Sigma Chemical Co.] and stained with APC-conjugated Annexin V for 15 min at room temperature. Annexin V fluorescence was determined with a Becton Dickinson FACScalibur or LSRII flow cytometer. Annexin V binds to those cells that express phosphatidylserine on the outer layer of their membrane (7). Patient-derived cells from patient samples were stained with PE-labeled anti-CD34 and Annexin V APC. The extent of apoptosis was quantified as percentage of Annexin V-positive cells, and the extent of drug-specific apoptosis was assessed by this formula: % specific apoptosis = (test - control) × 100 / (100 - control) (8).

Western blot analysis. Cells were lysed at a density of $1 \times 10^6/50$ µL in protein lysis buffer (0.25 mol/L Tris-HCl, 2% sodium dodecyl sulfate, 4% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and heated at 95°C for 10 min. The lysis buffer was supplemented with a protease inhibitor cocktail (Roche Diagnostic Co.). Cell lysates were then loaded onto a 10% to 12% SDS-PAGE gel (Bio-Rad). After electrophoresis, proteins were transferred to Hybond-P membranes (Amersham Pharmacia Biotech), followed by immunoblotting. Signals were detected using a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics).

Coimmunoprecipitation. Cells (10×10^6) were washed with 1× PBS and resuspended in ice-cold 1% CHAPS lysis buffer [150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), 1% CHAPS and protease inhibitors (Roche)] on ice for 30 min. Insoluble debris was removed by centrifugation at 4°C for 10 min at 13,000 rpm. Protein A-coated 96-well strips (Pierce) were washed thrice with CHAPS lysis buffer. For each 10^7 cells, 2.5 µg of antibody [Bcl-2/Bim co-IP, hamster anti-Bcl-2 antibody (BD); bak/bax co-IP, mouse antibody specifically recognizing conformationally changed bak (Calbiochem; ref. 9); Mcl-1/Bak/Bim co-IP, rabbit anti-Mcl-1 (Santa Cruz); control, mouse, hamster, or rabbit IgG (Santa Cruz)] was incubated in each well in 100 µL CHAPS lysis buffer with shaking for 1 h at room temperature. The strips were then washed thrice with CHAPS lysis buffer. The cell extracts (10×10^6 cell equivalent) were added to the antibody-bound wells

and shaken overnight at 4°C. The wells were washed five times with CHAPS lysis buffer. Immunoprecipitated proteins were solubilized from the protein A antibody wells with 2× SDS-PAGE sample buffer [0.25 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 4% β-mercaptoethanol, 0.02% bromophenol blue]. The samples were heated for 3 min by placing the well strip directly on a 95°C heating block. Proteins were separated by 12% SDS-PAGE gels, which were then transferred to Hybond-P membranes (Amersham Pharmacia Biotech) and detected by immunoblotting using rabbit anti-Bim (Calbiochem), mouse anti-Bcl-2 (Dako), rabbit anti-bak (Upstate), rabbit anti-bax (BD), mouse anti-bak (Calbiochem), or mouse anti-Mcl-1 (BD) antibodies. Signals were detected using a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics).

Mitochondrial cytochrome c release. HL60 cells were grown in T-175 flasks in RPMI 1640 supplemented with 10% FBS to a cell density of 5×10^5 cells/mL. 1×10^8 cells were collected by centrifugation and washed in 10 volumes of ice-cold PBS. Cells were resuspended in 10 volumes of ice-cold CEI buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA] and incubated on ice for 10 min. The swollen cell suspension was homogenized by forcefully passing through a 24-G needle six to eight times. One volume of cold CEI buffer (CEI buffer supplemented with 170 mmol/L sucrose, 440 mmol/L mannitol) was added to the cell suspension and gently mixed by inversion followed by centrifugation at 800 rpm for 5 min to collect nuclei and unbroken cells. The supernatant was then centrifuged at 3,500 rpm for 10 min, and the pellet was washed twice in cold CEI buffer. The mitochondrial pellet was resuspended in 500 µL of M buffer [125 mmol/L KCl, 20 mmol/L HEPES, 10 mmol/L Tris-Cl, 2 mmol/L KPO₄ (pH 7.2)] and maintained on ice. Protein was quantitated from 5 µL of a 1:5 dilution using the bicinchoninic acid method. The purity of the mitochondrial preparations was assessed by Western blot. Fractions were immunoblotted with COXIV and GAPDH to determine the presence of mitochondrial and cytosolic components, respectively (10). Using the above methodology, cross-contamination of cytosolic and mitochondrial fractions was not observed. Mitochondria were then resuspended in M buffer at 0.8 mg/mL protein and equilibrated at room temperature for 2 min before the addition of obatoclast. The concentration of DMSO in the solution did not exceed 0.2%. Mitochondrial suspensions were incubated for 15 min at room temperature, and mitochondria were collected by centrifugation at 14,000 rpm for 5 min. The presence of cytochrome c was evaluated by Western blotting of the mitochondrial pellet and the supernatant.

BrdUrd incorporation. Cells were incubated with BrdUrd (final concentration, 10 µg/mL) for 1 h at 37°C with 5% CO₂. Cells were then washed and fixed in cold ethanol. After treatment with Rnase, DNA was partially denatured with 2N hydrochloric acid for 20 min. Anti-BrdUrd antibody (BD Biosciences) and appropriate secondary FITC-conjugated antibody were added. Counterstaining for total DNA content was performed using propidium iodide.

AML blast colony and colony-forming unit-granulocyte-macrophage assays. AML bone marrow cells were isolated by gradient centrifugation and plated in duplicate at a density of 1 to 2×10^5 cells/mL in 1% methylcellulose in IMDM (Methocult; Stem Cell Technologies) containing 10% FBS and the following human recombinant growth factors: erythropoietin (3 units/mL), interleukin 6 (IL-6; 10 ng/mL), IL-3 (10 ng/mL), granulocyte-macrophage colony stimulating factor (10 ng/mL), and stem cell factor (50 ng/mL). Obatoclast was added at the start of cultures at concentrations of 50 to 100 nmol/L. In four experiments, mononuclear cells isolated from normal bone marrow (1×10^4 cells/mL) were plated, as described above. The colony-forming capacity of AML and normal samples was evaluated under a stereo or inverted microscope after 8 to 10 d of culture at 37°C in a 5% CO₂ humidified environment. A colony was defined as a cluster of 40 or more cells [blasts (colony-forming unit, CFU) or erythrocyte (blast-forming unit), granulocyte, monocyte (CFU-GM), or the mixed population (CFU-GEMM)].

Small interfering RNA transfection. Silencing of *Bim* and *Bak* gene expression in leukemic cells was achieved by the small interfering RNA (siRNA) technique. siRNAs were obtained as duplexes in purified and desalted form (option C) from Dharmacon. The sense strand of the siRNA silencing *bim* gene (Bim-siRNA) was GACCGAGAAGGUGACAAUUGdTdT. Bak ON-TARGETplus SMARTpool (L-003305-00) was purchased from

Dharmacon. Nonspecific control pool containing four pooled nonspecific siRNA duplexes was also used as a negative control (called NS-siRNA, Dharmacon-Upstate). Transfection of leukemic cells was carried out by electroporation using the Nucleofection system (Amaxa) following the manufacturer's instructions. Briefly, 2×10^6 cells were resuspended in 100 μ L of T-cell nucleofector solution containing 4,000 nmol/L of double-stranded siRNAs. After electroporation, 500 μ L of cultured medium were added to the cuvette, and the cells were transferred into culture plates containing 1.5 mL prewarmed culture medium (siRNA final concentration, 67 nmol/L).

Statistics. Results are expressed as means \pm SE of two to three replicates unless otherwise indicated. Synergism, additive effects, and antagonism were assessed with the Chou-Talay method (11) and Calcsyn software (Biosoft); the combination index (CI) for each experimental combination was calculated. When CI = 1, the equation represents the conservation isobologram and indicates additive effects. CI values of <1.0 indicate an additive effect characteristic of synergism.

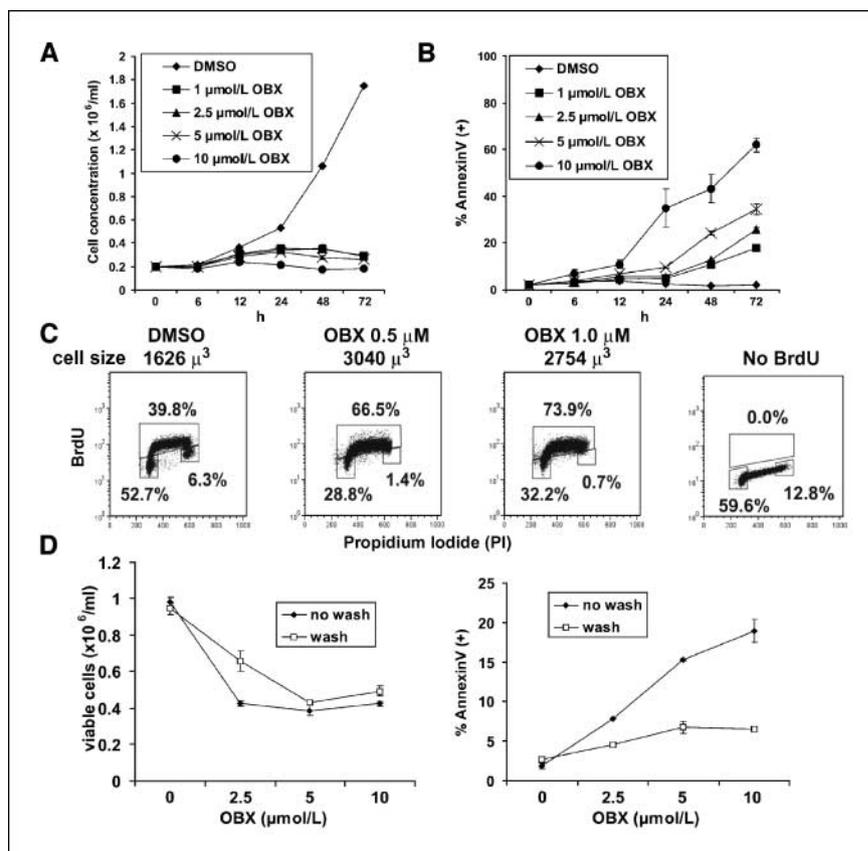
Results

Obatoclox induces apoptosis in AML cell lines. Obatoclox is a novel cycloprodigiosin-derived small molecule BH3 inhibitor that binds with moderate affinity to all antiapoptotic Bcl-2 family members, including Mcl-1, and is currently undergoing phase I clinical trials in leukemias (12). To evaluate the antileukemia effects of obatoclox, we incubated OCI-AML3 AML cells with various concentrations of obatoclox (0, 0.5, 1, 2.5, 5, and 10 μ mol/L) and measured the number of viable cells using a Vi-CELL XR cell viability analyzer (Fig. 1A). Our results suggested that obatoclox effectively abrogated the growth of OCI-AML3 cells, and similar results were seen in HL60, KG1, and U937 cells (not shown). To then determine if apoptosis contributes to the antiproliferative effect of GX15-070,

OCI-AML3 cells were treated with increasing concentrations of this agent for various times, and phosphatidyl serine externalization was monitored by flow cytometry by staining with Annexin V-APC. As shown in Fig. 1B, GX15-070 induced a time-dependent and dose-dependent externalization of phosphatidyl serine that was detected as early as 24 hours after exposure and at doses that paralleled the reported IC₅₀ values (1.1–5.0 μ mol/L) for antagonism of Bcl-2 family proteins (6). Interestingly, obatoclox also displayed low-dose antiproliferative properties that were accompanied by an S-G₂ cell cycle block as shown by an increase in BrdUrd-labeled S-phase cells concomitant with the disappearance of cells in G₂-M cell cycle phase, as well as an increase in cell size as determined by the Coulter ViCell XR analyzer (Fig. 1C). To investigate if the apoptotic and antiproliferative effects of obatoclox could be differentiated pharmacokinetically, we performed a washout experiment (Fig. 1D). The results show that wash out of obatoclox after 1-hour exposure prevents the induction of apoptosis (*right*) but does not prevent the observed growth inhibitory effects (*left*), suggesting that the targets that mediate apoptosis are different from those mediating mitotic arrest. Nonetheless, our observations suggest that, at concentrations of obatoclox that display affinity for antiapoptotic Bcl-2 proteins, apoptosis contributes to the observed antiproliferative effects. Similar results were also found in HL-60 cells (data not shown).

Obatoclox-induced apoptosis proceeds through the intrinsic apoptotic pathway after neutralization of Mcl-1. We have previously reported that the BH3 mimetic ABT-737 promotes the release of cytochrome *c* from isolated HL60 mitochondria (5). To investigate if obatoclox could exert similar effects, we exposed succinate/rotenone-energized HL60 mitochondria to obatoclox

Figure 1. Obatoclox induces apoptosis in AML. **A**, OCI-AML3 cells were incubated with various concentrations of obatoclox (0, 1, 2.5, 5, and 10 μ mol/L), and the number of viable cells was determined as described in the Materials and Methods. **B**, OCI-AML3 cells were treated with increasing concentrations of obatoclox for various times and phosphatidyl serine externalization was monitored by flow cytometry by staining with Annexin V-APC. **C**, OCI-AML3 cells were treated with 0.5 and 1.0 μ mol/L of obatoclox for 48 h, and BrdUrd incorporation was quantitated by flow cytometry as described in the Materials and Methods. Cell volume was determined from the average diameter measured by the ViCell XR analyzer. **D**, cells were treated with obatoclox for 1 h and washed twice in serum-containing media. Cells were then cultured under standard conditions for 48 h, and cell viability and apoptosis were quantitated as described in Materials and Methods.



(10 $\mu\text{mol/L}$) for 15 minutes followed by a cold-centrifugation step and assessed the levels of cytochrome *c* in the pellet and corresponding supernatant. As shown in Fig. 2A, obatoclax promotes the release of cytochrome *c* from isolated mitochondria, suggesting that, like ABT-737, this agent induces apoptosis through activation of the intrinsic apoptotic pathway. Similar results were obtained with U937 cell mitochondria (not shown). We then investigated if obatoclax-induced activation of the intrinsic pathway involved the release of Bak from the potent antiapoptotic protein Mcl-1, a protein that we have previously reported mediates resistance to ABT-737 (5). Treatment of OCI-AML3 cells with obatoclax resulted in a rapid and complete release of Bak from Mcl-1 (Fig. 2B), and this was accompanied by increased expression of a conformationally altered Bak in a complex with Bax (Fig. 2C; ref. 9). Additionally, it was observed that obatoclax-induced apoptosis was decreased, but not completely abolished, in Bak^{-/-} cells (Fig. 2D), suggesting that Bak contributes to some extent to cytotoxicity induced by this agent. No further protection from cell death was seen in Bax/Bak^{-/-} MEFs (not shown). Finally, we sought to determine if, similar to ABT-737-induced apoptosis, obatoclax-induced apoptosis proceeded in a Bim-independent manner in leukemia cells. We observed that Bim was efficiently released from Bcl-2 and Mcl-1 in OCI-AML3 cells treated with obatoclax (Fig. 3A and B), and most interestingly, cells devoid of Bim expression were less susceptible to apoptosis induction by this BH3 mimetic (Fig. 3C). These results suggest that cells treated with obatoclax-free Bim may cooperate with Bak to promote the activation of the intrinsic apoptotic pathway. Indeed, partial knockdown of both Bim and Bak by siRNA in HL-60 cells partially protected cells from apoptosis (Fig. 3D), whereas cells electro-

porated with Bak or Bim siRNA alone were minimally protected. Although we were unable to achieve complete knockdown in notoriously difficult-to-transfect leukemic cells, these data suggest other targets contributing to proapoptotic effects of this agent. In contrast, cell cycle analysis of wild-type, Bax-deficient, Bak-deficient, Bax/Bak-deficient, or Bim-deficient MEFs showed that obatoclax triggered an S-G₂ cell cycle block irrespective of the status of these proteins (data not shown).

Obatoclax synergizes with AraC and ABT-737 in inducing apoptosis in AML cell lines. Because the binding affinities of obatoclax to antiapoptotic Bcl-2 proteins are different than those of ABT-737 and each agent binds to a different but overlapping region of the hydrophobic pocket of Bcl-2, we sought to investigate if the combination of both BH3 mimetics could synergistically activate apoptosis. Briefly, ABT-737-resistant OCI-AML3 cells were treated simultaneously with ABT-737 and obatoclax using a fixed ratio (1:1) and Annexin V positivity was monitored by flow cytometry after 48 hours. Isobologram analysis (Fig. 4A) revealed that obatoclax and ABT-737 act synergistically in inducing apoptosis (averaged CI values, 0.3 ± 0.03) suggesting that indeed the combination of two BH3 mimetics with different binding characteristics promotes a greater degree of apoptosis than each agent alone. Furthermore, as shown in Fig. 4B, obatoclax also synergized with AraC, a frontline chemotherapeutic agent for the treatment of AML, to induce apoptosis in OCI-AML3 cells (fixed 1:1 ratio; averaged CI, 0.36 ± 0.04). Finally, pretreatment with AraC for 24 hours or pretreatment of obatoclax for 24 hours did not significantly alter the average CI values for 48-hour combination treatment with these agents, suggesting the schedule independence of their interaction (Supplementary Table S1). Similar results were

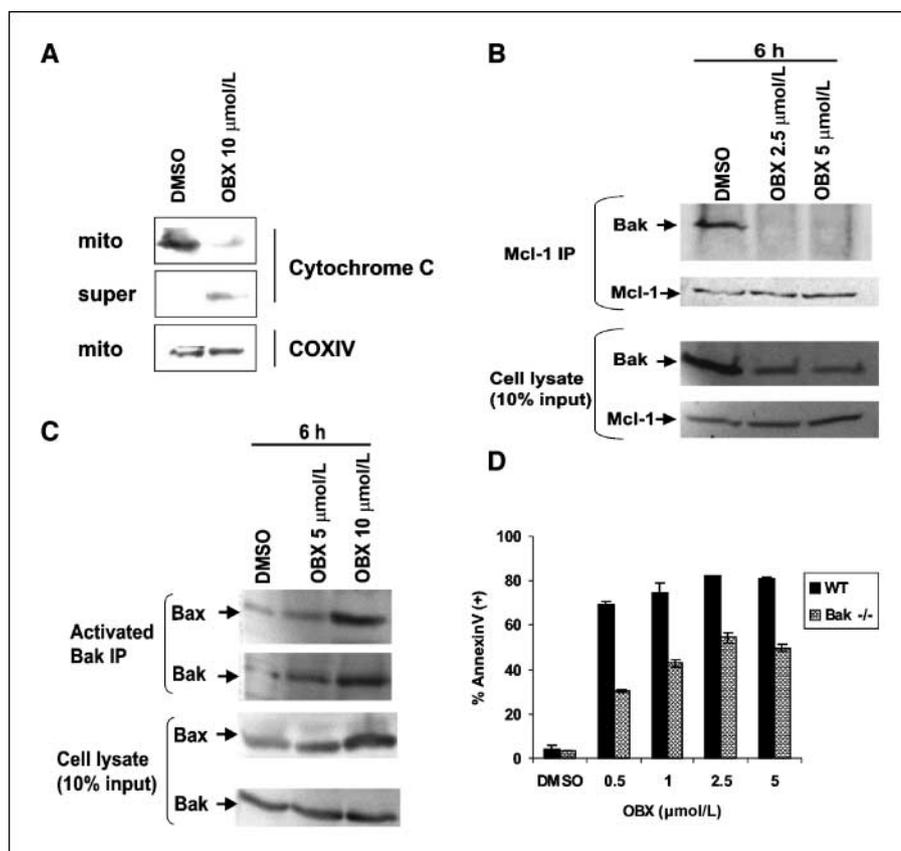
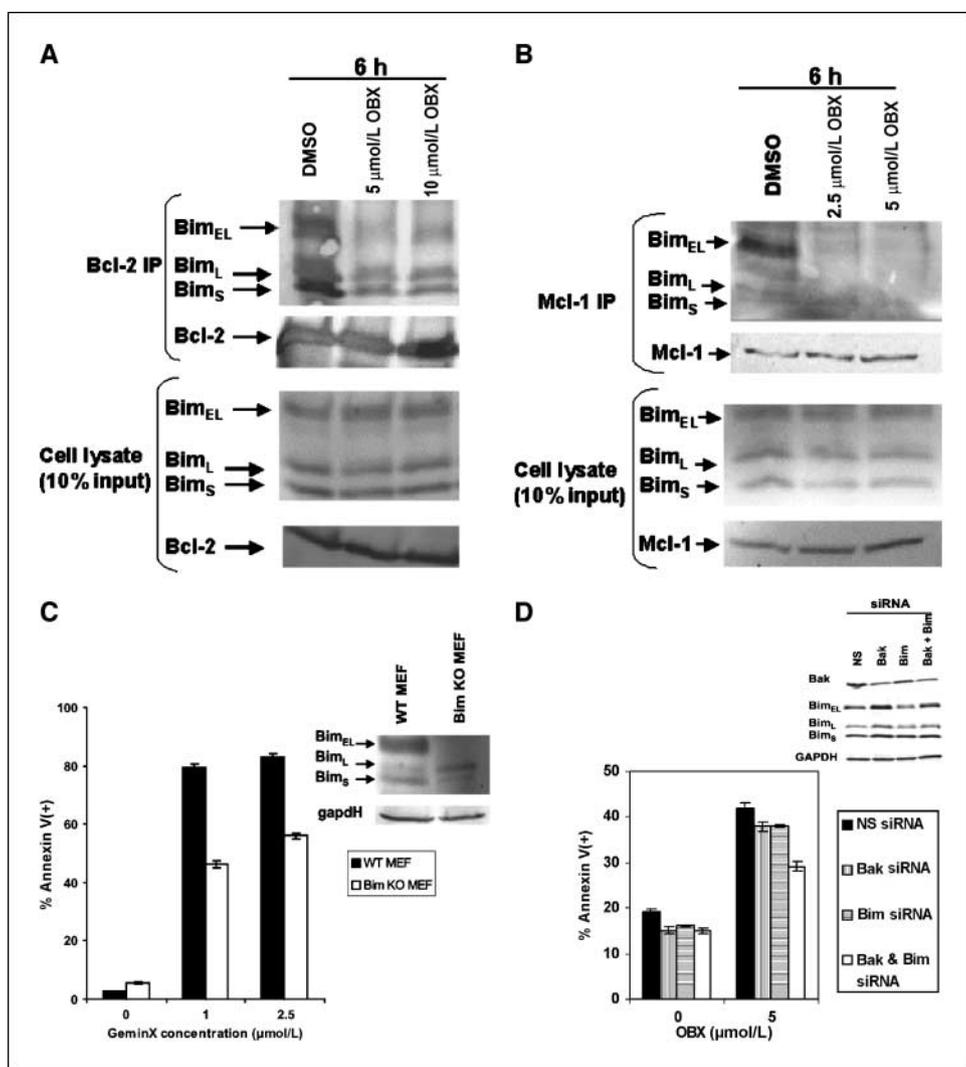


Figure 2. Obatoclax activates the intrinsic apoptotic pathway. *A*, succinate/rotenone-energized HL60 mitochondria were exposed to obatoclax (10 $\mu\text{mol/L}$) for 15 min and the levels of cytochrome *c* in the pellet and corresponding supernatant were determined as described in the Materials and Methods. *B*, Mcl-1 was immunoprecipitated from OCI-AML3 cells treated with obatoclax for 6 h, and the presence of Bak was determined by Western blot. *C*, conformationally changed Bak was immunoprecipitated from OCI-AML3 cells treated with obatoclax for 6 h using antibody that specifically recognizes NH₂ terminal epitope, and the presence of Bax was determined by Western blot. *D*, wild-type (WT)-deficient or Bak-deficient MEFs were treated with obatoclax for 48 h, and Annexin V positivity was monitored by flow cytometry as described in Materials and Methods.

Figure 3. Obatoclox induces release of Bim from antiapoptotic Bcl-2 and Mcl-1 proteins. *A*, Bcl-2 was immunoprecipitated from obatoclox-treated OCI-AML3 cells, and the presence of Bim was investigated by Western blot. *B*, Mcl-1 was immunoprecipitated from obatoclox-treated OCI-AML3 cells, and the presence of Bim was investigated as above. *C*, Bim-deficient MEFs were treated with obatoclox for 48 h, and Annexin V positivity was monitored by flow cytometry as described in Materials and Methods. *D*, Bak, Bim, Bak, and Bim siRNA or control (NS) siRNA was transfected into HL-60 cells using Amaxa nucleofection, and the levels of Bim/Bak expression were analyzed by Western blot. Cells were treated with 5 $\mu\text{mol/L}$ obatoclox for 48 h, and induction of apoptosis was assessed by Annexin V flow cytometry.

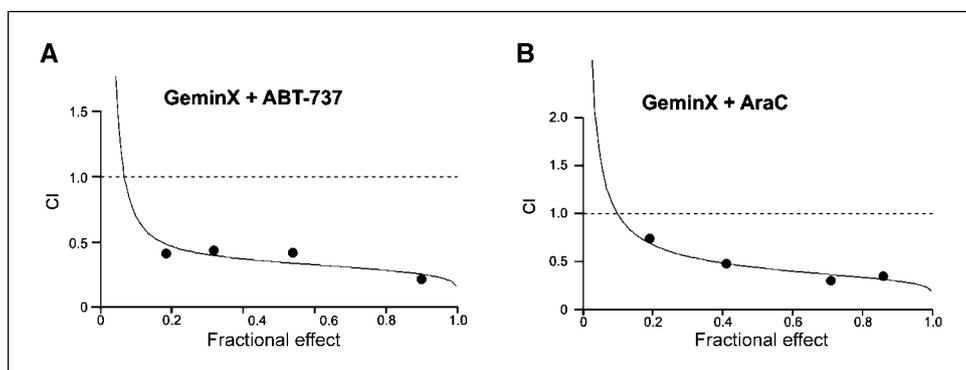


observed in HL60 cells, as well as in a primary AML sample, with averaged CI values for apoptosis induction of 0.062 and 0.43, respectively. These results suggest that, like other BH3 mimetics (5, 13, 14), obatoclox can potentiate the effects of traditional chemotherapy and may offer a therapeutic advantage in combination with other targeted agents.

Obatoclox induces apoptosis and selectively inhibits colony formation of primary AML cells. To determine the effects of obatoclox on AML progenitor cells, primary AML samples were

treated with increasing concentrations of obatoclox, and Annexin V was measured in the CD34-positive compartment by flow cytometry after 24 hours (Fig. 5A). All samples were obtained from untreated (newly diagnosed) or relapsed AML patients (Table 1A). Specific apoptosis was induced at 250 nmol/L obatoclox ($16.04 \pm 4.79\%$) and increased in a dose-dependent manner up to the highest dose tested (10 $\mu\text{mol/L}$, $73.48 \pm 8.39\%$; Supplementary Table S2). The average IC_{50} for the seven AML patient samples tested was $3.59 \pm 1.23 \mu\text{mol/L}$. Additionally, albeit Mcl-1 expression

Figure 4. Obatoclox synergizes with ABT-737 and AraC to induce cell death in OCI-AML3 cells. *A*, OCI-AML3 cells were treated simultaneously with ABT-737 and obatoclox using a fixed ratio (1:1), and Annexin V positivity was monitored by flow cytometry after 48 h and CI values were determined by isobologram analysis. *B*, ABT-737-resistant OCI-AML3 cells were treated simultaneously with AraC and obatoclox, and CI values were determined as above.



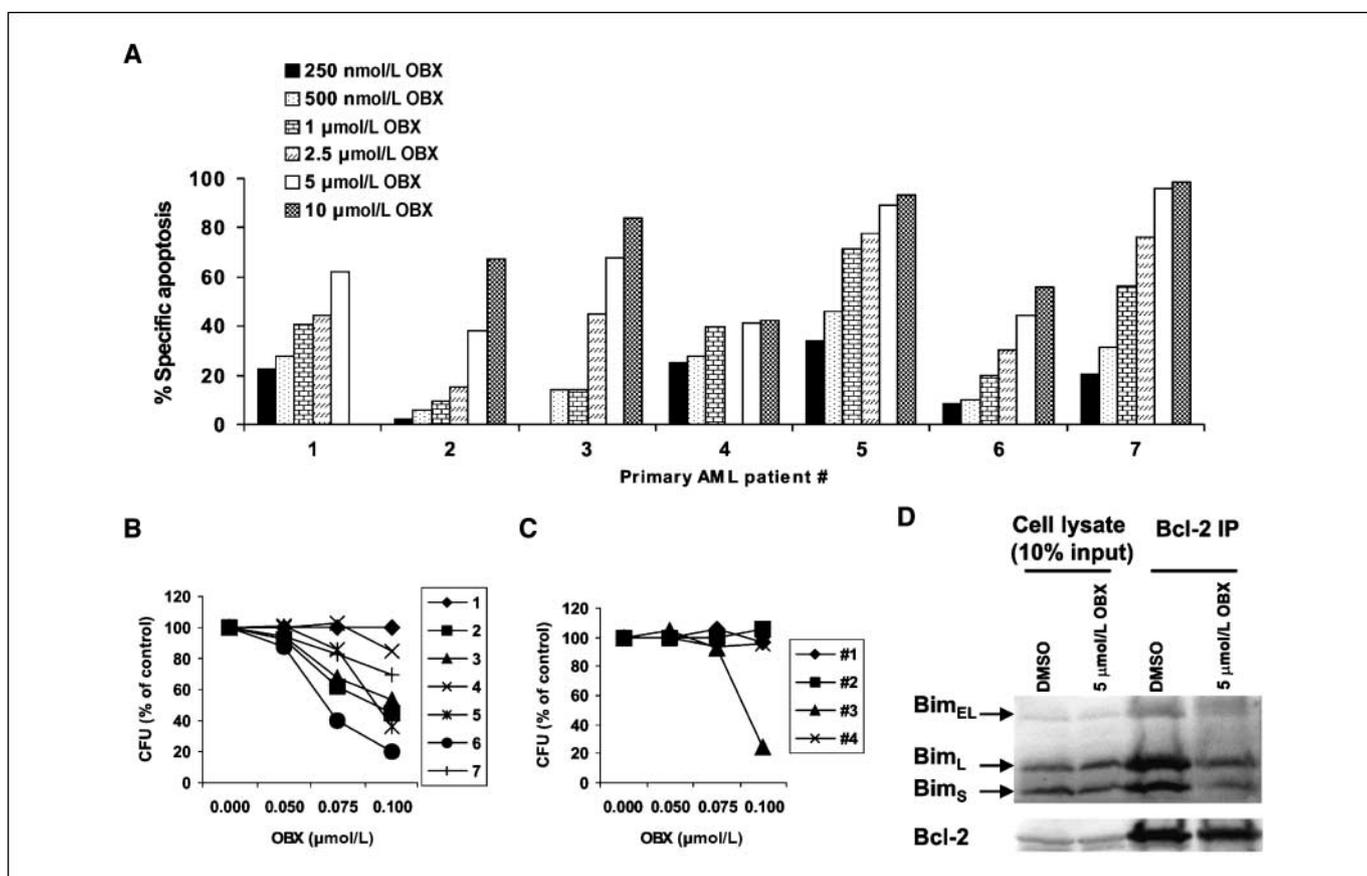


Figure 5. Obatoclox induces apoptosis in primary AML samples. **A**, primary AML samples were treated with increasing concentrations of obatoclox, and Annexin V was measured in the CD34-positive compartment by flow cytometry after 24 h. Specific apoptosis was determined as described in Materials and Methods. **B**, primary AML samples were cultured with obatoclox as indicated, and clonogenicity was determined as described in Materials and Methods. **C**, the clonogenic potential of normal bone marrow samples treated with obatoclox was determined as above. **D**, Bcl-2 was immunoprecipitated from a primary AML sample treated with obatoclox, and the presence of Bim was assessed by Western blot. Results are representative of three independent samples examined.

was very low in the primary samples examined, obatoclox was able to efficiently dissociate Bim from Bcl-2 in all three primary samples tested (Fig. 5D), suggesting that cell death induced by this agent in AML is associated with antagonism of Bcl-2. Furthermore, we investigated the effects of obatoclox on the clonogenicity of untreated or relapsed primary AML samples (Table 1B) in the CFU blast assay. The formation of surviving AML progenitor colonies was reduced to $77.33 \pm 8.41\%$ at 75 nmol/L and $58.45 \pm 10.63\%$ at 100 nmol/L (Fig. 5B). Colony inhibition in normal bone marrow was only reduced to $98.00 \pm 3.22\%$ at 75 nmol/L and $80.63 \pm 18.88\%$ at 100 nmol/L (Fig. 5C). The average IC_{50} for obatoclox in AML was $0.18 \pm 0.07 \mu\text{mol/L}$ and for normal bone marrow was $0.44 \pm 0.22 \mu\text{mol/L}$.

Discussion

The development of BH3 mimetics has offered a novel therapeutic approach for the treatment of cancer (15). We have previously reported that the BH3 mimetic ABT-737 efficiently induces cell death in AML cell lines and primary samples and preferentially targets AML progenitor cells. ABT-737 binds with high affinity to Bcl-2, Bcl-xL, and Bcl-w, but not to Mcl-1 or A1, and as such, it is ineffective in promoting cell death in cells that express Mcl-1, like OCI-AML3 cells. Albeit we observed that in certain cell contexts the expression of Mcl-1 can be diminished by pharma-

cologic inhibition of the mitogen-activated protein kinase (MAPK) pathway, resulting in sensitization to apoptosis induction by ABT-737, we are concerned that in certain cancer cells Mcl-1 expression may be independent of MAPK and therefore cannot be down-regulated by MAPK inhibitors. These cancer cell contexts will require the use of BH3 mimetics that can efficiently target Mcl-1 for therapeutic benefit. Additionally, from a mechanistic standpoint, investigating how direct Mcl-1 antagonism modulates apoptosis in leukemia cells, alone or in combination with other therapeutic approaches, is of utmost importance for the development of novel therapeutic approaches.

Here, we report that obatoclox (GX070-15), a BH3 mimetic currently in clinical trials that displays a different binding affinity than that of ABT-737 to antiapoptotic Bcl-2 family members, is effective in inducing apoptosis in AML cell lines and primary samples. Like ABT-737, obatoclox induced apoptosis in a time-dependent and dose-dependent manner and apoptosis induction occurred at doses that reflected the affinity of this agent for Bcl-2 family proteins. Mechanistically, apoptosis induction by obatoclox was preceded by liberation of Bak from Mcl-1, dissociation of Bim from Bcl-2 and Mcl-1, and the formation of a complex of conformationally altered Bak, previously reported to promote apoptosis (9), with Bax. Interestingly, the Bax and Bak complex formation induced by obatoclox has also been observed in peripheral blood mononuclear cells of patients with refractory

chronic lymphocytic leukemia within a single agent phase I trial. Unlike observed for ABT-737, apoptosis induced by obatoclox was diminished, but not abolished, in the absence of Bak/Bax, suggesting that additional target(s) other than Bcl-2 contribute to the activation of the intrinsic pathway by this cycloprodiginosin derivative. In addition, we identified that obatoclox can abolish

cell growth independently of apoptosis by inducing a S-G₂ cell cycle block. This antiproliferative effect could be temporally separated from the proapoptotic effects of obatoclox in washout experiments and occurred in the absence of Bax/Bak/Bim proteins, suggesting that this agent has multiple targets. Nevertheless, the abolishment of clonogenicity of primary AML samples treated with low doses of

Table 1. Clinical data for patients

Patient no.	Source	Blast, %	FAB	Cytogenetics	Status
A. Samples used for colony assays					
1	BM	47	RAEBT	46,XX,del(5)(q13q33)[2],42-46,XX,-2,-3,del(4)(q25q31.2),del(5)(q13q33),-6,-7,der(7;15)(q10;q10),+8,-12,-18,+20,+2-5mar[cp18]	Relapsed/refractory
2	BM	99	UNK	Pseudodiploid metaphase 46,XY,-3,add(12)(p13),+mar[1],Diploid male karyotype 46,XY[29]	Relapsed/refractory
3	BM	65	M2	Pseudodiploid clone 46,XX,t(6;9)(p23;q34)[19],Hyperdiploid metaphase 47,XX,t(6;9)(p23;q34),+16[1]	New
4	BM	88	M4	Pseudodiploid clone 46,XY,inv(16)(p13.1q22)[20]	New
5	BM	86	M1	Hypodiploid clone 45,XX,-7[15],Diploid female karyotype 46,XX[5]	Relapsed
6	BM	83	M1	42-44,Y,del(X)(p22.1),-3,add(4)(q35),-5,del(7)(q22q34),add(12)(p13),-17,der(18)del(18)(p11.2)del(18)(q21.1),add(21)(p11.1),+0-2mar[cp20]	Relapsed
7	BM	84	UNK	Pseudodiploid clone 46,XY,inv(5)(p15.1q13)[12],Metaphases exhibiting random numerical and structural changes[2],Diploid male karyotype 46,XY[6]	Relapsed/refractory
B. Samples used for <i>in vitro</i> assays					
1	PB	85	M1	Diploid female karyotype 46,XX[19]	New
2	PB	20	RAEBT	Pseudodiploid clone 46,XX,-7,+mar[18],Metaphases exhibiting random changes involving chromosome 7[2]	Primary/refractory during salvage
3	PB	90	M4	Two hypodiploid clones 45,XX,t(3;3)(q21;q26),-7[9],45,XX,inv(1)(p22q32),t(3;3)(q21;q26),-7[6]	Relapsed/refractory
4	PB	100	M1	Pseudodiploid clone 46,XY,t(12;17)(p13;q24)[20]	Relapsed/refractory
5	PB	81	M4	Hypodiploid clone 45,XY,-7[11],Diploid male karyotype 46,XY[9]	New
6	PB	93	M0	45,XX,del(1)(p13p31),-7,t(11;19)(q23;p13.3),del(12)(p13)[5],45,idem,add(6)(q27)[2],45,XX,t(1;3)(p36.1;p21),t(5;6)(q11.2;q11),-7,t(11;19)(q23;p13.3)[13]	Relapsed/refractory
7	BM	96	M1	Diploid male karyotype 46,XY[20]	New
8	PB	62	M5B	Pseudodiploid clone 46,X,idel(X)(p11.2)[4],Pseudodiploid metaphase 46,XX,t(1;4)(q42;q23)[1],Diploid female karyotype 46,XX[15]	New
9	PB	95	M2	Metaphases exhibiting random numerical and structural changes[4],Diploid male karyotype 46,XY[16]	New
10	PB	98	UNK	45,XY,t(3;3)(q21;q26.2),-7[11],43-45 XY,t(3;3)(q21;q26.2),del(4)(p14p16),-7,-8,-10,+0-5mar[cp8]	Refractory
11	PB	73	M4	Diploid female karyotype 46,XX[20]	New
12	PB	63	M1	Diploid male karyotype 46,XY[20]	New
13	BM	81	M4	Diploid female karyotype 46,XX[20]	New
14	PB	64	Raising from MDS	Pseudodiploid clone 46,XX,t(5;7)(q34;p13)[17],Pseudodiploid metaphase 46,XX,t(1;8)(p22;q22)[1],Diploid female karyotype 46,XX[2]	Relapse 2

Abbreviations: UNK, unknown (outside diagnosis); BM, bone marrow; PB, peripheral blood; FAB, French-American-British; RAEBT, refractory anemia with excess of blasts in transformation.

obatoclastox strongly suggests that the Bcl-2-independent targets of this agent may have clinical applicability. The mechanisms of these antiproliferative effects of obatoclastox require further studies which are outside of the scope of this manuscript.

From a therapeutic standpoint, we observed that obatoclastox could potentiate the activity of AraC, and most interestingly, we found that this agent synergized with ABT-737 to induce apoptosis. These findings suggest that this agent may not only augment the clinical activity of traditional chemotherapy, but it can potentiate the activity of other BH3 mimetics with different binding affinities/patterns. Interestingly, a recent report showed that DNA-damaging agents synergized with ABT-737 in killing of lung cancer cells via, in part, increased expression of Bim (16), suggesting that the observed synergy of obatoclastox with ABT-737 may be mediated not just by liberation of Bak from Mcl-1, but amplified by the near-

complete release of Bim from Mcl-1. In addition, we observed that in primary samples obatoclastox-induced apoptosis was associated with liberation of Bim from Bcl-2, suggesting the potential utility of this observation as a biomarker of activity in clinical trials with this agent. We propose that the combinatorial use of obatoclastox with chemotherapy and/or ABT-737 may offer a superior therapeutic benefit for AML patients via modulation of the apoptotic rheostat.

Acknowledgments

Received 5/23/2007; revised 1/25/2008; accepted 3/6/2008.

Grant support: NIH grants CA16672, CA49639, and CA55164.

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Cancer Res 2008;68:3413-3420.

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