A Bax-Mediated Mechanism for Obatoclax-Induced Apoptosis of Cholangiocarcinoma Cells

Rory L. Smoot, Boris R.A. Blechacz, Nathan W. Werneburg, Steve F. Bronk, Frank A. Sinicrope, Alphonse E. Sirica, and Gregory J. Gores

Abstract

Apoptosis induction by BH3 mimetics is a therapeutic strategy for human cancer. These mimetics exert single-agent activity in cells "primed" for cell death. Primed cells are dependent upon antiapoptotic Bcl-2 proteins for survival and are characterized by the ability of the BH3 mimic to induce cytochrome c release from their isolated mitochondria. Our aim was to examine the single-agent activity of obatoclax, a BH3 mimetic in cholangiocarcinoma cell lines. In clonogenic assays, inhibition of colony formation was observed by obatoclax treatment. Despite single-agent activity by obatoclax, the mitochondria from these cells did not release cytochrome c after incubation with this BH3 mimetic. However, immunofluorescence and cell fractionation studies identified Bax activation and translocation to mitochondria after treatment with obatoclax. shRNA targeted knockdown of Bax doubled the IC50 for obatoclax but did not abrogate its cytotoxicity, whereas knockdown of Bak did not alter the IC50. In a cell-free system, obatoclax induced an activating conformational change of Bax, which was attenuated by a site-directed mutagenesis of a previously identified protein activation site. Finally, the drug also elicited a significant in vivo response in a rodent model of this disease. In conclusion, single-agent obatoclax treatment results in Bax activation, which contributes, in part, to cell death in cholangiocarcinoma cells. These data indicate that BH3 mimetics may also function as direct activators of Bax and induce cytotoxicity in cells not otherwise primed for cell death. Cancer Res; 70(5); 1960–9. ©2010 AACR.

Introduction

Oncogene and multiple stress pathways active in human cancers predispose malignant cells to death by apoptosis (1). As a result, cancers often acquire mechanisms to evade apoptosis to develop and progress (2). Apoptosis is regulated by Bcl-2 family proteins, a protein family consisting of three subsets: prosurvival proteins, such as Mcl-1, Bcl-2, A1, and Bcl-XL; the proapoptotic multidomain proteins Bax and Bak; and BH3-only proteins, such as Bim, Bid, Bad, Noxa, Bmf, Hrk, Bik, and PUMA (3). The BH3-only proteins act as initiators of the core cell death machinery. These proteins may be classified as activators, which by directly triggering Bax or Bak activation induce mitochondrial dysfunction and cellular demise, or sensitizers, which sensitize the cell to apoptotic cues by inhibiting the function of prosurvival Bcl-2 proteins. Bim, Bid, and possibly PUMA are classified as activators, whereas the other BH3-only proteins are viewed as sensitizers (4–6).

Given the ability of the BH3-only proteins to initiate cell death pathways, the pharmaceutical industry has developed an array of BH3-only protein mimetics (7). However, to date, none of these has proved to be a direct activator. The binding profiles of these mimetics are not uniform, and resistance to these drugs has been documented. For example, overexpression of Mcl-1 is now a recognized mechanism of resistance to the BH3-only protein mimetic ABT-737, as this mimetic specifically binds Bcl-2 and Bcl-XL, but not Mcl-1 (8, 9). Obatoclax, a recently developed BH3 mimetic, has a different binding specificity than ABT-737, as it binds to all prosurvival members of the Bcl-2 protein family, including Mcl-1 (10). This property of obatoclax provides a potential therapeutic advantage for this drug.

ABT-737, which functions as a sensitizing BH3 mimetic, exerts single-agent activity in selected malignant cell lines despite its inability to directly activate Bax or Bak. Work by Letai and coworkers suggests that the apparent paradox of direct cytotoxicity by a sensitizing BH3 mimetic in the absence of additional, exogenously applied apoptotic stimuli can be explained if certain cells are “primed” for cell death (11). The mitochondrial antiapoptotic proteins of such cells are near capacity in their ability to sequester the activator BH3-only proteins Bim, PUMA, and Bid. Additional BH3-only proteins or mimetics displace preexisting bound activators from this sequestration, liberating them to trigger Bax or Bak oligomerization on the mitochondrial membrane (12).
The isolated mitochondria of such cells release cytochrome c in the presence of a BH3 mimetic (13). In contrast, the mitochondria of nonprimed cells are not “loaded” with activator BH3-only proteins bound to antiapoptotic proteins, and isolated mitochondria from these cells do not release cytochrome c upon exposure to a BH3 mimetic.

Direct Bax activation is a common mechanism for cell death by proapoptotic stimuli (14). Bax exists in the cytosol as an inactive globular protein. Recent data suggest that activator BH3-only proteins may directly activate Bax by binding to a site distinct from its BH3 groove and mutation of lysine 21 (K21E) abolishes Bax activation (15). After activating signals, the protein undergoes a conformational change, exposing both its NH2 terminal and COOH terminal domains (16). This conformational activation facilitates its translocation to mitochondria, where its COOH terminal transmembrane domain inserts into the outer mitochondrial membrane. Further stimuli promote its homooligomerization forming channels or pores in the mitochondrial outer membrane (14, 17). These pores result in the egress of cytochrome c and other proapoptotic proteins from the intermitochondrial membrane space into the cytosol, triggering cell death cascades.

Cholangiocarcinoma, a devastating neoplasm (18), is resistant to current medical therapies likely due to abundant Mcl-1 expression (19). Because obatoclax binds Mcl-1 in addition to other antiapoptotic Bcl-2 proteins, we examined this BH3 mimetic as a potential therapeutic agent for cholangiocarcinoma. In these studies, obatoclax was observed to exert single-agent activity in human cholangiocarcinoma cell lines, as well as in a syngeneic rat orthotopic model of cholangiocarcinoma. Despite the single-agent activity, the mitochondria of these cells were not “primed” for cell death. Instead, obatoclax treatment was associated with Bax activation, suggesting that obatoclax may function as a direct activator of this proapoptotic protein.

Materials and Methods

Obatoclax. Obatoclax was supplied by GeminX Biotechnologies, Inc. For in vitro studies, obatoclax was dissolved at 20 mmol/L in DMSO and aliquoted. Aliquots were further diluted in sterile water immediately before each experiment. The maximal DMSO concentration was 0.1% (v/v), which was used as a vehicle control in respective in vitro experiments. For in vivo experiments, obatoclax was prepared according to the supplier’s protocol.

Cell lines and culture. The human cholangiocarcinoma cell lines HuCCT-1 (20), KMCH (21), and Mz-ChA-1 (22) and the rat cholangiocarcinoma cell line BDeneu (23, 24) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, and 100 μg/L insulin.

Clonogenic and cell death assays. Clonogenic assays were performed, as previously described (25). For colony quantification, six-well plates were stained with coomassie blue and colonies were counted using ImageJ software (26) The IC50 was calculated as indicated using CalcuSyn software (Bio-soft). Apoptosis was confirmed by examining nuclear morphology using fluorescent microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI), as previously described (27).

Immunoblot analysis. Whole-cell lysates were prepared as previously described in detail (25), and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blotted with primary antibody at the indicated dilutions. Antibodies used were Bcl-2 (N-19 Santa Cruz, 1:1,000), Bcl-XL (S-18 Santa Cruz 1:1,000), Bak (N-20 Santa Cruz, 1:5,000), Bim (BD Pharmigen 1:500), Bid (R&D Systems 1:1,000), Noxa (ProSci 1:500), PUMA (Sigma 1:1,000), and actin (C-11 Santa Cruz 1:1,000). Horseradish peroxidase–conjugated secondary antibodies for mouse (Invitrogen), rabbit (Invitrogen), and goat (Biosource) were used at a concentration of 1:2,000. Proteins were visualized with enhanced chemiluminescence reagents (Hygro Quick Spray, Denville Scientific) and Kodak X-Omat film.

Mitochondria isolation. Mitochondria were isolated from cells by nitrogen cavitation and differential centrifugation, as previously described (28). Briefly, cells were lifted from a minimum of five 150-mm culture plates (~90% confluent) in MA buffer [100 mmol/L sucrose, 1 mmol/L EGTA, 20 mmol/L MOPS (pH 7.4), 1 g/L bovine serum albumin (BSA), and complete protease inhibitors (Roche Diagnostics)] followed by centrifugation at 800 × g for 10 min. Pellets were resuspended in 3 mL of MA buffer, and nitrogen cavitation was completed (420 lb/in2 for 10 min) on ice. The cell lysate was centrifuged at 2,500 × g for 5 min to remove cellular debris. The clarified supernatant was centrifuged at 16,000 × g for 15 min to pellet mitochondria. The pellet was resuspended in MA buffer, and protein concentration was quantified via the Bradford assay.

Cytochrome c release assay. Isolated mitochondria (100 μg of protein per reaction) were resuspended in 50 μL of MA buffer. Vehicle, drug, tBid (20 nmol/L), or lysis buffer was added, and the reaction was incubated at 37°C for 30 min. After incubation, the suspension was centrifuged at 16,000 × g for 15 min. The mitochondrial pellet and supernatant were separated. Samples were subjected to immunoblot analysis for cytochrome c (Santa Cruz, 1:1,000).

Bax immunofluorescence. Immunofluorescence for the active conformation of Bax was performed as previously described by us (29). The Bax 6A7 antibody (Exalpha, 1:100) was used as a primary antibody, and secondary staining was performed with an Alexa 633–conjugated antitmouse antibody (1:1,000, Invitrogen). Images were obtained using an inverted confocal microscope (LSM510; Carl Zeiss Microimaging, Inc.) equipped with a 40× lens using LSM510 imaging software (Carl Zeiss Microimaging, Inc.) using excitation and emission wavelengths of 633 and 656 nm/L, respectively.

Bax mitochondrial translocation, insertion, and oligomerization. For Bax translocation, mitochondria were isolated from cells as described above (five 150-mm dishes at 90% confluency per condition). The mitochondria were resuspended in lysis buffer on ice for 15 min, and after centrifugation at 14,000 × g for 15 min, the supernatant was
resolved by SDS-PAGE. Immunoblot analysis for Bax (N-20 Sigma, 1:5,000) and, as a control for protein loading, cytochrome c oxidase (Sigma, 1:1,000) was completed.

For Bax mitochondrial insertion, mitochondria were isolated from cells as described above (five 150-mm dishes at 90% confluency per condition). After protein concentration determination, 500 μg of mitochondrial protein were resuspended in 500 μL of 0.1 mol/L Na2CO3 (pH 12; Sigma) and incubated on ice for 20 min. The suspension was then centrifuged at 100,000 × g for 1 h to collect mitochondrial membranes. The pellet was resuspended in 500 μL MB buffer [210 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EDTA, 10 mmol/L HEPES (pH 7.5)] with 2% CHAPS, incubated on ice for 1 h, sonicated, and centrifuged at 100,000 × g for 30 min. The supernatant (integral membrane proteins) was analyzed by SDS-PAGE and immunoblot analysis for Bax (N-20 Sigma, 1:5,000). VDAC (Rockland, 1:1,000) served as a loading control for integrated proteins.

**Bax activation in a cell-free system.** Recombinant human Bax was prepared as previously described using the IMPACT system (New England Biolabs; ref. 30). The recombinant human Bax construct was a generous gift from Dr. Nico Tjandra. Protein concentration was determined using Bradford reagent (Sigma). A 20-μL reaction of recombinant Bax protein (300 ng) in column buffer [20 mmol/L HEPES, 500 mmol/L NaCl (pH 8.5)] was incubated with vehicle, increasing concentrations of obatoclax or 5% NP40 detergent at 37°C for 1 h. After incubation, 2 μL were removed from each reaction to assay as an input, and then 250 μL of 3% BSA in PBS were added to each reaction with 15 μL of 6A7 anti-Bax antibody (Santa Cruz) and incubated for 1 h at 4°C. Protein A agarose beads (60 μL) were then added to each reaction, and the mixture was incubated at 4°C overnight. The beads were pelleted, washed with PBS, and boiled in SDS sample buffer. The supernatant was subjected to immunoblot analysis for Bax (N-20 Santa Cruz, 1:2,000). Site-directed mutagenesis of the lysine 21 residue to glutamic acid in the Bax construct was completed using the QuikChange II kit (Stratagene). The K21E product was confirmed by sequencing, and protein preparation and reaction were undertaken as above. Oligomerization was assayed by incubating 5 μg of recombinant protein in column buffer (50-μL reaction), with vehicle or increasing concentrations of obatoclax at 37°C for 1 h. Bis[sulfosuccinimidyl] suberate (Thermo Scientific) was then added at a concentration of 0.25 mmol/L for 30 min at room temperature. The reaction was quenched.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Obatoclax has single-agent activity against cholangiocarcinoma, which is not associated with altered BCL-2 protein expression. A, cell death was quantified by clonogenic assay in four cholangiocarcinoma cell lines (HuCCT-1, Mz-ChA-1, BDEneu, KMCH-1) treated with obatoclax at doses ranging from 0 to 800 nmol/L for 48 h. Results are colony forming units as a percentage of control and represent means from three separate trials. B, immunoblotting of three obatoclax-sensitive cholangiocarcinoma cell lines treated with vehicle or increasing doses of obatoclax (100–400 nmol/L) for 24 h. *a*, no commercially available antibody for rat Mcl-1.

---

Cancer Res; 70(5) March 1, 2010 Cancer Research

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2010 American Association for Cancer Research.
with 20 mmol/L Tris-HCl (pH 8.0) at room temperature for 15 min, and the reactions were assayed by immunoblotting with the Bax N-20 antibody.

**Bax and Bak targeted knockdown by shRNA.** shRNA constructs for Bax and Bak were generated and validated, as previously described (31). HuCCT-1 cells were stably transfected with the shRNA constructs for Bax and Bak using Fugene HD reagent (Roche). Cells were selected with puromycin (0.5 mg/L) in normal growth medium. Knockdown of Bax and Bak expression was confirmed in clonal populations by immunoblot analysis. Cells were then used in clonogenic assays, as previously described.

**In vivo studies.** All animal experimentation described in this study was performed in accordance with and approved by the Institutional Animal Care and Use Committee. In vivo cell transplantation was carried out in adult Fischer 344 male rats (Harlan) with initial mean body weights ranging between 200 and 250 g, as previously described in detail by us (32). For survival and tumor size studies, a subcutaneous port (Instech Laboratories) was implanted on the low back of the rodent (through the subcutaneous port) once daily for five consecutive days starting 10 d after tumor implantation. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were carried out using the *In situ* Cell Death Detection kit (Roche) according to the supplier’s protocol, as previously described by us (32). Slides were viewed on an epifluorescence microscope (Nikon Eclipse TE200, 60× objective), and positive cells were counted in 10 high-power fields.

**Statistical analysis.** Data represent at least three independent experiments using cells from a minimum of three separate isolations and are expressed as means ± SDs. Differences between groups were compared using two-tailed Student’s *t* tests or *χ*² tests. Survival was analyzed by Kaplan-Meier method, and groups were compared by log-rank analysis.

**Results**

**Single-agent activity of obatoclax in cholangiocarcinoma cells.** Clonogenic assays were undertaken to examine the single-agent activity of obatoclax in four cholangiocarcinoma cell lines: three human cell lines HuCCT-1, KMCH, and Mz-ChA-1 and the rat cholangiocarcinoma cell line BDEneu. The HuCCT-1, Mz-ChA-1, and BDEneu cell lines were sensitive to obatoclax inhibition of colony formation in a concentration-dependent manner (Fig. 1A). A >50% inhibition of colony formation was observed at concentrations between 5 and 100 nmol/L in all three cell lines with complete inhibition observed at 200 nmol/L obatoclax. The cholangiocarcinoma cell line KMCH was not sensitive to obatoclax as a single agent at these doses, consistent with our previously published observations (25). The sensitivity of the human cholangiocarcinoma cell lines was directly proportional to the level of Mcl-1 expression, with KMCH cells containing the highest level of Mcl-1 expression (Supplementary Fig. S1). Obatoclax concentrations of 400 nmol/L and greater did inhibit colony formation of KMCH cells, suggesting that the cytoprotective effect of Mcl-1 can be overcome with higher drug concentrations (data not shown). Because either cell cycle arrest or apoptosis can inhibit colony formation in clonogenic assays, apoptosis by obatoclax was confirmed in BDEneu cells (Supplementary Fig. S2). In this context, we have shown that obatoclax displays single-agent activity against several cholangiocarcinoma cell lines.

**Cholangiocarcinoma mitochondria are not primed for death.** To exclude altered expression of proapoptotic or antiapoptotic Bcl-2 proteins as a mechanism for cell death by obatoclax, immunoblot analysis for these proteins was performed. No consistent changes in expression of the Bcl-2 family proteins were observed with obatoclax treatment in any of the three obatoclax-sensitive cell lines (Fig. 1B). Given that expression of the Bcl-2 family proteins was unaltered in obatoclax-treated cells, we sought to further examine the mechanism for single-agent cytotoxicity by this BH3 mimic. As single-agent activity by obatoclax suggests that cholangiocarcinoma cells are primed for cell death, we next examined the ability of this BH3 mimic to
directly induce cytochrome c release in isolated mitochondria. Unexpectedly, addition of obatoclax to mitochondria isolated from all three sensitive cholangiocarcinoma cell lines failed to directly induce cytochrome c release, although cytochrome c was readily identified in the supernatant when the mitochondria were incubated with tBid protein or lysed by detergent (Fig. 2). These data suggest that the mitochondria from these cholangiocarcinoma cell lines are not primed for death, but rather obatoclax-induced mitochondrial dysfunction occurs through a more complex cascade involving cytosolic components.

**Obatoclax treatment leads to Bax activation and translocation to mitochondria.** Bax is a cytosolic protein, which potently triggers cell death upon its activation. Because BH3 proteins and therefore potentially a BH3 mimetic may directly activate Bax (33, 34), we examined the activation and subcellular localization of Bax after treatment with obatoclax. Bax activation by obatoclax was by immunofluorescence for an active conformation of Bax, using the 6A7 antibody, in all three cholangiocarcinoma cell lines (Fig. 3A). A 3-fold increase in mitochondria-associated Bax was also observed in isolated mitochondria after obatoclax treatment (Fig. 3B). The translocation of Bax to mitochondria was associated with its insertion into the mitochondrial membrane, as the association was resistant to bicarbonate treatment (Fig. 3C). Collectively these data suggest that obatoclax either directly or indirectly promotes Bax activation.

**Direct activation of Bax by obatoclax.** To ascertain whether the activation of Bax by obatoclax may be via direct mechanism, recombinant Bax was incubated with obatoclax in a cell-free reaction. After incubation with obatoclax, the active conformation of Bax could be immunoprecipitated with the Bax 6A7 antibody (Fig. 4A). The amount of active Bax recovered with obatoclax treatment increased in a concentration-dependent manner. This conformational change in Bax was not observed in vehicle-treated protein; however, treatment with the detergent NP40, which induces activating-like conformational changes in Bax, also lead to recovery of active Bax with the 6A7 antibody. To further show the specificity of this activation, site-directed mutagenesis of the lysine 21 residue K21E was performed. This residue has previously been shown to be an important residue in a Bax activation site (15). The activation of the K21E Bax was attenuated as shown by a decrease in the amount of immunoprecipitated active Bax after obatoclax treatment compared with detergent-treated protein (Fig. 4A). Additionally, oligomerization of wild-type Bax (trimers and tetramers) in the cell-free system could be shown in a concentration-dependent manner with obatoclax treatment and cross-linking (Fig. 4B); however, the higher-order multiples were significantly decreased or absent in the mutant protein. Unexpectedly, a protein complex consistent with Bax dimerization was observed with cross-linker alone. In this cell-free system, obatoclax treatment promotes Bax activation.

**Cell death by obatoclax is facilitated by Bax.** To further elucidate the contribution of Bax in obatoclax-induced apoptosis, HuCCT-1 cells stably expressing shRNA for Bax or Bak were generated. The specificity and efficiency of the shRNA knockdown of the respective targets in these cells was confirmed by immunoblot analysis (Fig. 5A). The median dose effect ($D_{50}$) for obatoclax-induced cell death, a calculation of the median effect (surrogate for LD$_{50}$ in cell death assays), was compared between wild-type cells and those in which
Bax or Bak had been selectively knocked down. For wild-type HuCCT-1 cells, the $D_m$ was 27.0 nmol/L and the Bak knockdown cells had a similar $D_m$ of 21.8 nmol/L. In contrast, the $D_m$ for obatoclax doubled to 55.3 nmol/L in HuCCT-1 cells stably expressing the shBax construct, indicating a protective effect by reducing Bax expression (Fig. 5B). These data provide further evidence suggesting Bax contributes to obatoclax-induced cell death.

In vivo activity of obatoclax. Given the in vitro activity of obatoclax against cholangiocarcinoma cell lines, we sought to test the in vivo effects of this drug using a recently characterized syngeneic rat orthotopic model of cholangiocarcinoma. Tumors were removed after treatment with obatoclax or vehicle for 5 days. TUNEL staining showed a significant increase in the number of cells undergoing apoptosis in tumors removed from obatoclax-treated rats compared with controls (12.3 ± 1.9 versus 5.8 ± 3 cells per high power field, $P = 0.05$) with no increase in apoptosis in normal hepatocytes (1 ± 0.1 versus 0.9 ± 0.2 cells per high power field, $P = 0.5$). To further examine the in vivo efficacy of obatoclax, the drug was given via a hepatic artery injection to ensure adequate tumor exposure to the drug. An initial group of rats ($n = 8$) had their livers removed 5 days after completing treatment, whereas a second group was observed for survival ($n = 12$). The mean tumor weights in animals treated with obatoclax was 10-fold lower than vehicle-treated controls (0.04 ± 0.03 versus 0.38 ± 0.10 g, $P < 0.001$). This relationship also held true when the ratio of tumor weight to liver weight was calculated (Fig. 6A); a 12-fold decrease in this ratio was observed in obatoclax-treated animals compared with controls (0.003 ± 0.002 versus 0.036 ± 0.010, $P < 0.001$). This decrease in tumor size also correlated with increased survival.
in obatoclax-treated rats (Fig. 6B). The median survival increased from 23 days in control animals to 44 days in obatoclax-treated animals \(P = 0.005\).

**Discussion**

The results of this study provide new insight into the mechanisms of obatoclax-mediated cell death. The results indicate that obatoclax \((a)\) exerts single-agent activity in cholangiocarcinoma cell lines, \((b)\) induces cell death by promoting Bax activation, and \((c)\) is efficacious in an in vivo model of cholangiocarcinoma. Each of these results is discussed in greater detail below.

In the current study, we observed maximal single-agent obatoclax activity between concentrations of 50 to 100 nmol/L in two human cell lines and at 200 nmol/L in a third rat cell line (Fig. 1A). Obatoclax has been tested in small, phase I studies of patients with advanced hematologic malignancies (35, 36). In these studies, multiple dosing regimens were tried with doses ranging from 3.5 to 40 mg/m². The reported recommended phase II dose was 28 mg/m², which corresponds to a mean maximal plasma concentration of 73 ng/mL when infused over a 3-hour period (35). This peak plasma concentration is equivalent to \(\sim 175\) nmol/L. Thus, biologically active concentrations of obatoclax observed in our in vitro studies are achievable at doses used in humans.

Obatoclax, as a single agent, inhibited colony formation in clonogenic assays. Although cell cycle arrest will also manifest as an inhibition of colony formation in this assay, we also confirmed the occurrence of apoptosis as shown by morphology and dependence on Bax. The BH3 mimetic ABT-737 also exerts single-agent activity in selected malignant cell lines (37). These cells are under BH3-only protein stress, as the mitochondria isolated from such cell lines are already "loaded" with BH3-only proteins bound to antiapoptotic proteins. The activator BH3-only proteins released from their sequestration by the BH3 mimetic then induce mitochondrial dysfunction with cytochrome c release (11). However, we failed to observe this phenomenon with obatoclax in mitochondria isolated from our cell lines. We, therefore, examined the possibility that the cells are not under BH3-only protein stress but that obatoclax delivers a sufficient activating BH3 stimulus for the cells to undergo apoptosis. If this was true, the stimulus should directly result in Bax or Bak activation. Interestingly, we observed Bak activation in obatoclax-treated cells as manifest by an activating conformational change, translocation, and insertion into mitochondria. These data suggest that obatoclax provides a sufficient BH3 stimulus to promote Bax activation. As Mcl-1 can antagonize Bax activation (38), it is likely that the ability of obatoclax to bind Mcl-1 also contributes to Bax activation in these cells.

Although Bak and Bax are often thought to be functionally redundant proteins in mediating outer mitochondrial membrane permeabilization during apoptosis (39), several observations suggest specificity for signaling through one or the other in a cell type–specific, context-specific, and stimulus-specific manner (40, 41). Our studies are consistent with a preferential specificity for Bax in obatoclax-mediated cell death in cholangiocarcinoma cell lines. For example, Bax knockdown by shRNA doubled the median dose effect for obatoclax (Fig. 5B). The cell-free system studies and activation assays also suggest that obatoclax has the potential to promote activating conformational changes in Bax. To our knowledge, this is the first study to suggest that a BH3-only protein mimetic may function as a direct activating stimulus for Bax activation. This observation distinguishes obatoclax from ABT-737, which, like the BH3 domain peptide of Bad, only binds the prosurvival Bcl-2 proteins Bcl-2 and Bcl-XL (37) and acts as a sensitizing, but not activating, BH3 stimulus. Obatoclax treatment lead to significant apoptotic stimulating activity, decreased tumor size, and improved survival in our in vivo syngeneic, orthotopic rat model of cholangiocarcinoma (24). Not only does this model reflect a similar molecular signature as human cholangiocarcinoma but the syngeneic, orthotopic model avoids the problems of immunocompromise and incompatibilities of the tumor microenvironment problematic in...
human xenograft models. Analogous to our in vitro studies, obatoclax showed single-agent activity in vivo suggesting that the in vitro studies are applicable to the more complex native environment of these cancers.

Whereas our studies suggest that obatoclax can directly activate Bax in cell-free systems and cholangiocarcinoma cell lines, it is unlikely that this is the only mechanism for obatoclax-induced cell death. Previous studies have shown the ability of obatoclax to induce cell death in Bak/Bax double-deficient mouse embryonic fibroblasts (25, 42). Likely, there are as yet to be determined additional mechanisms for obatoclax-induced cell death.

In summary, obatoclax displays single-agent activity in human cholangiocarcinoma cells and a rodent model of cholangiocarcinoma that mimics the human disease. The mechanism of cell death seems to be at least partially dependent...
upon Bax activation, and the data support a process where obatoclax seems to directly activate this proapoptotic protein. These data extend our knowledge by which BH3 agonists promote cell death in cancer cells. Development of BH3 mimetics, which directly activate Bax, seems to be feasible. Finally our analyses also suggest that obatoclax warrants further evaluation for the treatment of human cholangiocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Acknowledgments

We thank Erin Nystuem-Bungum for excellent secretarial support and Dr. Scott H. Kaufmann for carefully reviewing and editing the manuscript.

Grant Support

NIH grants R01 DK9427 (G.J. Gores), R01 CA 83650, and R01 CA 39225 (A.E. Sirica); optical microscopy core of PO DK84570; Mayo Clinic Clinician Investigator Program (R.L. Smoot) and Mayo Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/23/2009; revised 11/25/2009; accepted 12/15/2009; published OnlineFirst 02/16/2010.

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2010 American Association for Cancer Research.
transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. Hepatology 2009;50:1861–70.


A Bax-Mediated Mechanism for Obatoclax-Induced Apoptosis of Cholangiocarcinoma Cells

Rory L. Smoot, Boris R.A. Blechacz, Nathan W. Werneburg, et al.

Cancer Res  Published OnlineFirst February 16, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-3535

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/02/15/0008-5472.CAN-09-3535.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.