BH3-only protein mimetic obatoclax sensitizes cholangiocarcinoma cells to Apo2L/TRAIL-induced apoptosis

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Abstract
Human cholangiocarcinomas evade apoptosis by overexpression of Mcl-1. The drug obatoclax (GX15-070) inhibits antiapoptotic members of the Bcl-2 family including Mcl-1. The purpose of this study is to determine if obatoclax sensitizes human cholangiocarcinoma cells to apoptosis. The human cholangiocarcinoma cell lines, KMCH, KMBC, and TFK, were employed for these studies. Protein expression was assessed by immunoblot and protein-protein interactions detected by coprecipitation of the polypeptide of interest with S-tagged Mcl-1. Activation of Bak and Bax was observed by immunocytochemistry using conformation-specific antisera. Obatoclax induced minimal apoptosis alone; however, it increased apoptosis 3- to 13-fold in all three cancer cell lines when combined with Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Obatoclax did not alter cellular expression of Bid, Bim, Puma, Noxa, Bak, Bax, Mcl-1, or cFLIP. Mcl-1 binding to Bak was readily identified in untreated cells, and this association was disrupted by treating the cells with obatoclax. Additionally, Bim binding to Mcl-1 was markedly decreased by obatoclax treatment. We also identified alterations in Bak and Bax conformation following treatment with obatoclax plus Apo2L/TRAIR but not with either Apo2L/TRAIR or obatoclax alone. In conclusion, obatoclax releases Bak and Bim from Mcl-1 and sensitizes human cholangiocarcinoma cells to Apo2L/TRAIR-induced apoptosis. Obatoclax is a potentially promising adjunctive agent for the treatment of this cancer. [Mol Cancer Ther 2008;7(8):2339–47]

Introduction
Cholangiocarcinoma, a neoplasm arising from the biliary tract, is the second most common primary hepatobiliary cancer and its incidence is increasing in western societies (1). Other than surgical extirpation, therapy has limited efficacy (2). Previous studies have shown that this cancer is resistant to therapy because of its ability to evade apoptosis as illustrated by its resistance to the death ligand Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; ref. 3); Apo2L/TRAIR is also known as TNFSF10 and is distinct from the Apo2L/TRAIL receptors DR4/Apo2/TRAIL-R1/TNFRSF10A and DR5/TRAIL-R2/TNFRSF10B, here referred to as DR4 and DR5, respectively. Overexpression of Mcl-1, a potent antiapoptotic member of the Bcl-2 family, plays a prominent role in apoptosis resistance on Apo2L/TRAIR treatment in cholangiocarcinoma cells (4) as indicated by the observation that targeted knockdown of Mcl-1 by small interfering RNA (4), cyclin-dependent kinase inhibition (4, 5), or anti-Mcl-1 microRNA silencing (6) restores Apo2L/TRAIR sensitivity. Of note, neither Bcl-2 nor Bcl-xL small interfering RNA caused sensitization to Apo2L/TRAIR. Further, Mcl-1 is overexpressed in 70% of primary tumor samples as measured by immunohistochemistry (7). Accordingly, approaches to inhibit Mcl-1 function would be predicted to help sensitize this cancer to conventional therapeutics or even promote tumor-autonomous death signaling in vivo given the propensity of this cancer to express Apo2L/TRAIR (3).

Mcl-1 blocks apoptosis by preventing the mitochondrial dysfunction that results from activation of the multidomain, proapoptotic members of the Bcl-2 family, Bax and Bak (8). According to current models, activated Bax and/or Bak form homo-oligomers within the outer mitochondrial membrane that cause membrane permeabilization and release of apoptogenic, intermembrane space proteins such as cytochrome c and Smac/DIABLO (9). Once in the cytosol, these polypeptides act through caspase-9 to promote the activation of the effector caspase-3 and caspase-7, which induce cell death.

The precise mechanisms responsible for Bax and Bak activation remain incompletely understood (10). There are two commonly discussed models of activation by BH3-only proteins, termed indirect and direct activation. In the indirect model, activated BH3-only proteins bind to Mcl-1 (or other antiapoptotic Bcl-2 family proteins) causing displacement of Bak and allowing Bak activation by release of Mcl-1 inhibition (11). In the direct model, activated BH3-only proteins directly bind and activate Bak and Bax, whereas Mcl-1 acts to bind and neutralize the BH3-only proteins. The activation of both Bax and Bak involves a conformational change exposing the NH2-terminal regions...
of both polypeptides, observed by immunoreactivity to conformation-specific antisera (12, 13). Additionally, both proteins are thought to form dimers and higher-order multimers, which act to permeabilize the mitochondrial membrane.

Given the critical role of BH3-only proteins in mediating cell death, BH3 mimetics would seem to be promising agents to restore the cell death program in cancers (14, 15). Recent studies have shown that ABT-737, a BH3 mimic that binds Bcl-2 and Bcl-xL but not Mcl-1 (16), restores sensitivity of Bcl-2-overexpressing cells but not Mcl-1-overexpressing cells to apoptotic stimuli (17, 18). Another BH3 mimic, obatoclax, is less well characterized but has been shown to inhibit Mcl-1 in addition to Bcl-2 and Bcl-xL, and is predicted to bind in the BH3-binding pocket of Bcl-2 (19, 20). Obatoclax is an indole bipyrrole compound that is being investigated as an anticancer agent in phase I and II clinical trials on hematologic and lymphoid malignancies as well as small cell and non-small cell lung cancers. Direct binding studies have been hampered by the hydrophobic nature of obatoclax, but an analogue compound Gx-15 has been studied in fluorescence polarization assays and competes for a fluorescently labeledBid BH3-peptide binding to Mcl-1 as well as Bcl-2 and Bcl-xL (21). After treatment with obatoclax, other groups have observed changes in expression of the BH3-only proteins Bim (19, 21, 22) and Noxa (23), both of which are known to interact with Mcl-1 and may contribute to apoptosis induction. Because obatoclax disables Mcl-1, we sought to determine whether obatoclax sensitizes human cholangiocarcinoma cell lines to Apo2L/TRAIL-mediated cytotoxicity. Because the cell lines (in contrast to primary cholangiocarcinoma tumor samples) do not express Apo2L/TRAIL (3), exogenous Apo2L/TRAIL was added to mimic the in vivo expression of Apo2L/TRAIL. Our results indicate that obatoclax sensitizes human cholangiocarcinoma cells to Apo2L/TRAIL by inhibiting Mcl-1 binding to Bak and Bim.

Materials and Methods

Cell Lines and Culture

Cholangiocarcinoma cell lines derived from mixed intrahepatic cholangiocellular-hepatocellular carcinoma KMCH (24) or extrahepatic cholangiocarcinoma KMBC (25) and TFK-1 (26) were used. Cholangiocarcinoma cell lines and mouse embryonic fibroblast (MEF) cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL gentamicin. KMCH cells stably transfected with S-peptide-tagged Mcl-1 (5) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamicin, and 1,200 µg/mL G418.

S-Peptide Pull-down

KMCH cells stably transfected with S-peptide-tagged Mcl-1 were incubated for 16 h with or without 0.5 µmol/L obatoclax. Apo2L/TRAIL (1 ng/mL) was added where indicated for 8 h. After treatment, cells were washed with PBS and lysed at 4°C for 1 h in lysis buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 1 tablet Complete protease inhibitors/50 mL (Roche Diagnostics), 1 mmol/L Na3VO4, 100 mmol/L NaF, 20 mmol/L microcystin, and 2% (v/v) CHAPS. After the protein concentration was determined by the Coomassie blue binding method (Pierce), aliquots containing 1 mg protein in 1 mL lysis buffer were incubated with 50 µL S-protein agarose beads (Novagen) at 4°C overnight. After at least six washes with lysis buffer, samples were released from the beads by boiling for 5 to 10 min in 50 µL Laemmli sample buffer (Bio-Rad Laboratories) containing 5% 2-mercaptoethanol. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes.

To visualize Noxa binding to Mcl-1, a stable cell line derived from KMCH that expresses short hairpin RNA against endogenous Mcl-1 and also a short hairpin RNA-resistant phosphomimetic serine to glutamic acid (S64E) substituted S-tagged Mcl-1 protein was used based on difficulty showing Noxa immunoreactivity in pull-downs using wild-type human S-tagged Mcl-1. Our previous investigations suggested that Noxa binds much more strongly to S64E Mcl-1 than the unphosphorylatable S64A protein (5). The use of the S64E protein is biologically relevant, as mouse and rat Mcl-1 wild-type proteins contain a glutamic acid at this position. Otherwise, S-peptide pull-down experiments for Noxa binding were as above.

Immunoblot Analysis

Whole-cell lysates were obtained by incubating cells on ice with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 tablet Complete protease inhibitors/50 mL (Roche Diagnostics), 1 mmol/L Na3VO4, 100 mmol/L NaCl, 1% NP-40, and 0.25% sodium deoxycholate]. Insoluble particulates were removed by centrifugation at 14,000 × g for 15 min at 4°C. Samples were resolved on 4% to 20% gradient SDS-PAGE, transferred to nitrocellulose membrane, and blotted with the indicated primary antibodies at a dilution of 1:1,000. Peroxidase-conjugated secondary antibodies (Biosource International) were incubated at a dilution of 1:3,000. Bound antibodies were visualized using enhanced chemiluminescence reagents (Amersham) and Kodak X-OMAT film. Primary antibodies used were those raised to Bak (Upstate), Bax (Santa Cruz Biotechnology), cFLIP (Pro-Sci), caspase-8 (BD Biosciences), DR4 (Pro-Sci), DR5 (Pro-Sci), Bim (BD Biosciences), Puma and Noxa (Pro-Sci), Mcl-1 (Santa Cruz Biotechnology), and actin (Santa Cruz Biotechnology).

Cell Death Assays

Apoptosis was quantified by assessing the characteristic nuclear changes of apoptosis (chromatin condensation and nuclear fragmentation) using fluorescence microscopy after staining with 4’,6-diamidino-2-phenylindole (Sigma). Caspase-3/7 activity in cell cultures was assessed using the Apo-ONE homogenous caspase-3/7 kit (Promega) per the supplier’s protocol.
Obatoclax sensitizes cholangiocarcinoma cells to Apo2L/TRAIL cytotoxicity

Previous studies from our laboratory have shown that human cholangiocarcinoma cells are resistant to Apo2L/TRAIL (4, 5) and sensitivity to this death ligand can be restored by down-regulation of Mcl-1 (4, 6) but not Bcl-2 or Bcl-xL (4). To build on these results, we examined the effect of obatoclax, a BH3 mimetic that targets Mcl-1 as well as Bcl-2 and Bcl-xL, on Apo2L/TRAIL sensitivity in the cholangiocarcinoma cell lines KMBC, KMCH, and TFK-1. All three lines were relatively resistant to apoptosis induced by exposure to the BH3 mimetic obatoclax or to Apo2L/TRAIL alone (Fig. 1). However, pretreatment with obatoclax sensitized all three cell lines to Apo2L/TRAIL-mediated apoptosis (Fig. 1A and B). Time-course studies showed that apoptotic changes were evident by 4 h and increased at 8 h (Fig. 1C). Because KMCH cells underwent Apo2L/TRAIL-induced apoptosis at obatoclax concentrations as low as 50 nmol/L (Fig. 1D), this cell line was employed further for mechanistic studies.

Obatoclax does not alter total cellular expression of antiapoptotic or proapoptotic proteins

Sensitization of cell lines to Apo2L/TRAIL can be mediated by increased expression of proapoptotic mediators or decreased expression of antiapoptotic mediators. We therefore next examined whole-cell lysates from KMCH cholangiocarcinoma cells treated for 16 h with obatoclax for expression of the Apo2L/TRAIL receptors DR4 and DR5, caspase-8, and cFLIP as well as Bcl-2 family members Bid, Bim, Noxa, Mcl-1, Bak, and Bax. As examined by immunoblot analysis, no significant change in steady-state cellular levels of these proteins was observed (Fig. 2A).

Obatoclax inhibits the association between Mcl-1 and Bak and Bim

Mcl-1 has been shown to inhibit apoptosis in part by binding and sequestering the proapoptotic multidomain Bcl-2 protein Bak (11), an association that is antagonized by BH3-only proteins (27). Therefore, we next determined if the BH3-mimetic obatoclax also diminishes binding of Bak to Mcl-1. KMCH cells stably expressing S-peptide epitope-tagged Mcl-1 were used because the S-peptide/S-protein high-affinity interaction provides a convenient and efficient method to selectively enrich for Mcl-1 and Mcl-1-bound polypeptides (5). Following S-peptide pull-down from CHAPS-lysed cells, Mcl-1 binding partners were assessed by immunoblot. In untreated KMCH cells, pull-down of S-peptide-tagged Mcl-1 coprecipitated Bak (Fig. 2B). In cells treated with obatoclax, reduced amounts of Bak were found associated with S-peptide-tagged Mcl-1. This is consistent with a BH3-mimetic mode of action that displaces Bak from Mcl-1. Using this technique, we did not observe binding of Bax to Mcl-1 (data not shown).

Apo2L/TRAIL-mediated cell death in KMCH cells is dependent on the BH3-only protein Bim as determined using small interfering RNA silencing of Bim (28). Importantly, Bim coprecipitated with Mcl-1 and obatoclax treatment (in the presence or absence of Apo2L/TRAIL) prevented this interaction (Fig. 2B). Thus, obatoclax interferes with binding of Bak and Bim, both proapoptotic, to Mcl-1.

The proapoptotic BH3-only protein Noxa specifically antagonizes Mcl-1 (11) and its involvement in obatoclax sensitization was sought. No change in Noxa protein expression was observed after 16 h of obatoclax treatment (Fig. 2A). In addition, Noxa binding to Mcl-1 as tested by coprecipitation with S-tagged Mcl-1 was diminutive and difficult to visualize (data not shown). We then tested a...
Figure 1. Obatoclax sensitizes cholangiocarcinoma cell lines to Apo2L/TRAIL killing. A, KMCH cancer cells were pretreated with 0.5 μmol/L obatoclax or vehicle overnight followed by obatoclax + Apo2L/TRAIL (1 ng/mL) for the final 8 h where indicated. Cells were fixed and stained with the nuclear dye 4',6-diamidino-2-phenylindole followed by imaging by confocal microscopy. Bar, 25 μm. B, cholangiocarcinoma cancer cell lines KMBC, KMCH, and TFK-1 were treated as in A and then stained with 4',6-diamidino-2-phenylindole followed by fluorescence microscopy. Apoptotic and normal nuclei were quantitated and apoptotic cells were expressed as a percent of total. Cells treated in parallel were assayed for caspase-3/7-like activity (DEVDase activity), which is expressed in arbitrary units. For statistical analysis, treatments were compared with vehicle. C, following pretreatment with diluent or obatoclax, KMCH cells were treated with or without Apo2L/TRAIL (1 ng/mL) for the indicated times followed by quantitation of apoptosis and caspase-3/7 activity. For statistical analysis, treatments were compared with vehicle at the corresponding time point. D, KMCH cells were pretreated with the indicated concentration of obatoclax followed by diluent (open symbol) or Apo2L/TRAIL (1 ng/mL; filled symbol) for 8 h. Apoptosis and caspase-3/7 activity were measured as above. Points, mean of triplicate experiments; bars, SE. For statistical analysis, Apo2L/TRAIL + obatoclax was compared with obatoclax alone at the corresponding concentration. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (A–D).
phosphomimetic Mcl-1 protein (S64E) shown previously to bind to Noxa (5). S-peptide pull-down of S64E Mcl-1 revealed Noxa binding was unchanged in the presence or absence of obatoclax (Fig. 2B).

**Obatoclax Sensitization to Apoptosis Does Not Depend on Pretreatment**

Treatment with obatoclax alone had little effect on cell death overnight; thus, in most of the experiments presented here, cells were pretreated overnight with obatoclax followed by the addition of Apo2L/TRAIL after 16 h. However, the suggested mechanism of action of obatoclax to occupy the BH3-binding pocket of antiapoptotic proteins should not require pretreatment. To test this, we added obatoclax (0.5 μmol/L final) to cells at 16, 4, and 1 h before Apo2L/TRA1 treatment (1 ng/mL) as well as adding both Apo2L/TRA1 and obatoclax simultaneously. Irrespective of the preincubation time, obatoclax sensitized to Apo2L/TRA1 treatment equally (Fig. 2C). In addition, the proposed mechanism of action should not depend on de novo transcription/translation of an apoptogenic protein. To test this, we inhibited protein synthesis (cycloheximide, 20 μg/mL) at the same time obatoclax was added (overnight treatment) and then added Apo2L/TRA1 for 8 h. The inhibition of protein synthesis in contrast to a protective effect actually enhanced killing by Apo2L/TRA1 alone or in combination with obatoclax (data not shown). Thus preventing translation did not decrease the injury, suggesting that new protein synthesis of proapoptotic proteins is not necessary for obatoclax activity.

**Obatoclax-Facilitated Killing Is Associated with Bak and Bax Activation**

The proapoptotic proteins Bak and Bax undergo a conformational change on activation that can be visualized using conformation-specific antibodies (12, 13). We tested the effect of obatoclax in the absence and presence of Apo2L/TRA1 on Bak and Bax NH2-terminal conformational alterations. Because initial studies showed that obatoclax exhibits fluorescence at fluorescein and rhodamine wavelengths but not at the far red end of the spectrum, we employed secondary antibodies labeled with the Alexa 633 fluorophore (λexcitation and λemission of 633 and 650 nm, respectively). Bak conformational changes were observed in the majority of cells treated with Apo2L/TRA1 plus obatoclax but not cells treated with diluent, Apo2L/TRA1, or obatoclax (Fig. 3A). The number of cells positive for staining with the conformation-specific Bak antiserum is consistent with the number of cells undergoing apoptosis (Fig. 1B). Similarly, Bax conformational changes were observed in cells treated with obatoclax plus Apo2L/TRA1 but not in the majority of cells treated with diluent or either stimulus alone (Fig. 3B).

**Obatoclax Killing Is Partially Dependent on Bax or Bak**

The activation of Bak and Bax in cells treated with obatoclax plus Apo2L/TRA1 is suggestive but does not show that obatoclax cytotoxicity is dependent on Bak or Bax. We tried several small interfering RNA constructs unsuccessfully to knock down Bax and/or Bak in KMCH cells. Therefore, to directly test the mechanism by which obatoclax sensitizes cells to apoptosis, we employed MEFs doubly deficient in Bak and Bax (DKO). Cell death was quantified in clonogenic assays, a rigorous method for assessing cell survival. This assay determines long-term survival after treatment, as cells must survive and proliferate to form colonies; colonies, not individual cells, are then counted after 10 days in culture. Wild-type MEFs were sensitive to obatoclax plus Apo2L/TRA1 treatment and, in fact, were sensitive to obatoclax as a single agent (Fig. 4). Single-agent activity suggests that wild-type MEFs are under chronic BH3 stress that is mitigated by Bcl-2 family members, and when treated with obatoclax, the wild-type MEFs can no longer resist apoptosis. DKO MEFs, on the other hand, were partially resistant to apoptosis induction by either obatoclax alone or combined obatoclax plus Apo2L/TRA1. Thus, cell killing depends, at least in part, on Bak/Bak protein expression.

**Discussion**

BH3 mimetics represent a new therapeutic tool in the treatment of human cancers (14). These agents have been shown by competition studies and X-ray crystallography to bind antiapoptotic proteins in the Bcl-2 family. For instance, the BH3 mimetic ABT-737 competes with a BH3 peptide (derived from Bad) for binding to Bcl-2, Bcl-xL, and Bcl-w but not Mcl-1 nor A1 (16). Indeed, Mcl-1 expression is now recognized as a common mechanism for ABT-737 resistance (17, 18). Obatoclax is a structurally dissimilar molecule that antagonizes Mcl-1 function in addition to Bcl-2 and Bcl-xL (19, 20). Further, the related compound GX-15 competes for BH3-peptide binding to Mcl-1, Bcl-2, and Bcl-xL using a fluorescence polarization assay (21). In recent studies, obatoclax has been reported to synergize with the proteasome inhibitor bortezomib in lymphoma cell lines (29), with tyrosine kinase inhibitors in breast cancer cells (30), and with cisplatin in non-small cell lung cancer cells (31). Our studies extend these observations by showing that obatoclax increases the cytotoxicity of Apo2L/TRA1 in cholangiocarcinoma cells. Because obatoclax does not appreciably kill these cells by itself, the enhanced effects of the combination meet the definition of synergy, as put forth by Berenbaum (32). This synergy is consistent with several observations by us and others that Mcl-1 is a key mediator of Apo2L/TRA1 resistance (4, 6, 33). This is also the first report, to our knowledge, showing that obatoclax can potentiate death ligand-induced apoptosis.

The precise mechanism of action of BH3-only proteins remains unsettled. One model posits that selected BH3-only proteins directly activate Bax and Bak (34), whereas another suggests that BH3-only proteins bind and inactivate antiapoptotic Bcl-2 family members, thereby allowing release of inhibition of Bak and Bax (35). Treatment of cells with obatoclax alone did not induce activation of Bax and Bak (measured as an increase in immunoreactivity to...
conformation-specific antisera; Fig. 3), making it unlikely obatoclax is a direct agonist of these proapoptotic proteins. Release of Bak and Bim binding to Mcl-1, however, was observed following obatoclax treatment (Fig. 2), consistent with obatoclax binding to Mcl-1 and disabling its anti-apoptotic function. The lack of Bak activation despite its release from Mcl-1 by obatoclax (cf. Figs. 2B and 3A) suggests that release from Mcl-1 is insufficient to induce full activation of Bak. These observations are consistent with the report that Bak mutants that do not bind Mcl-1 are not spontaneously lethal but still require further activation to induce cell death (36). We also observed Bak activation by Apo2L/TRAIL in obatoclax-treated cells, and Bax-dependent killing by Apo2L/TRAIL has also been reported (37). Because we were unable to get a >50% knockdown of Bax and Bak in these cells,3 we were unable to determine whether Bak and/or Bax is the principal mediator of cell death in cells treated with obatoclax and Apo2L/TRAIL.

The effector causing activation of Bak after release from Mcl-1 is unknown, but recent studies in our laboratory showed that Apo2L/TRAIL-dependent killing of KMCH cholangiocarcinoma cells is dependent on the BH3-only protein Bim (28). Interestingly, in addition to small interfering RNA against Bim, the JNK pathway inhibitor SP600125 also prevented Apo2L/TRAIL killing. Thus, it is reasonable to postulate that Apo2L/TRAIL induces activation of Bim through JNK-dependent phosphorylation (38). This activated Bim can still be held in check by Mcl-1, which may also act by neutralizing Bak. However, Apo2L/TRAIL treatment in the presence of obatoclax, which causes release of Bim and Bak from Mcl-1, facilitates apoptotic signaling (Fig. 5). Thus, Apo2L/TRAIL alone does not cause apoptosis as Bim (potentially phospho-Bim) and Bak are bound to Mcl-1. Similarly, obatoclax alone does not result in apoptosis, because the release of unactivated Bim and Bak does not cause death. The combination, though, results in release of Bak and Bim, and activation of Bim, resulting in Bak conformational change and mitochondrial permeabilization. We recognize that this mechanism of action (direct activation of Bak) is contentious. Indeed,

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3S.F. Bronk and G.J. Gores, unpublished observations.
alternative mechanisms of Bak activation remain possible. However, our data are inconsistent with spontaneous Bak activation on release from Mcl-1, as obatoclax treatment alone induced Bak release (Fig. 2B) but not Bak conformational change (Fig. 3) or apoptosis (Fig. 1).

Other groups have described alterations in BH3-only protein levels after obatoclax treatment (19, 22, 23). In cholangiocarcinoma cells, we do not see these changes after 16 h of treatment. Thus, there may be cell type–specific differences in the transcriptional/translational response to obatoclax. Additional evidence that increased protein synthesis is not required for Apo2L/TRAIL sensitization in cholangiocarcinoma cells includes the observation that pretreatment is not necessary for the full effect (Fig. 2C). Preventing protein synthesis with cycloheximide led to increased apoptosis, further suggesting that de novo protein synthesis is not required for death signaling with this combination; indeed, newly synthesized proteins (possibly the short-lived antiapoptotic protein cFLIP) may instead play a protective role.

Although many molecules have been reported to function as BH3 mimetics, several of these agents kill cells deficient in both Bax and Bak at the same efficiency as wild-type cells (18). In contrast, it has been reported that obatoclax activity is dependent on Bax and/or Bak, with baby kidney epithelial cells lacking Bax and Bak resistant to oligosomal DNA fragmentation and caspase-3 cleavage after obatoclax treatment compared with wild-type cells (19). We found partial protection of Bax/Bak DKO fibroblasts from obatoclax using a clonogenic assay. Of note, there was an unexpected decrease in colony-forming ability of the DKO MEFs on treatment with obatoclax. This may represent nonspecific (Bax/Bak independent) killing by obatoclax. This decrease was significantly lesser in wild-type MEFs (53% survival for DKO versus 8% survival for wild-type MEFs). What sets obatoclax apart from ABT-737, another agent that is a bona fide BH3 mimic, is the ability of the former to antagonize the antiapoptotic function of Mcl-1 as well as Bcl-2 and Bcl-xL.

Figure 3. Bax and Bak undergo conformational change on obatoclax and Apo2L/TRAIL treatment. A, KMCH cells were untreated or pretreated with 500 nmol/L obatoclax with or without Apo2L/TRAIL (1 ng/mL) treatment for the final 8 h and immunostained with antisera specific for the NH2 terminus of Bak. Minimal staining was observed in untreated or Apo2L/TRAIL-treated cells, whereas obatoclax-treated cells showed a slight increase in background fluorescence. Cells showing NH2 terminus accessible Bak exhibited high levels of fluorescent staining, readily distinguished from background. Bar, 25 μm. Cellular fluorescence was quantitated (Zeiss LSM software) as the average signal within manually outlined cells and corrected by subtracting the average fluorescence of cells stained with secondary antibody only. To correct for residual fluorescence from obatoclax, signal for obatoclax and Apo2L/TRAIL + obatoclax was corrected by subtracting the average fluorescence of cells pretreated with obatoclax and stained with secondary antibody only. Random cell fields were photographed and fluorescence was quantitated for all cells (n = 31–75 cells per condition). **, P < 0.01, compared with untreated cells (ANOVA with Tukey’s least significant difference post-test). B, immunofluorescent staining with a monoclonal antibody (6A7) specific for the Bax NH2 terminus was done similar to the experiments in A. Again, there was a slight increase in background with obatoclax alone, but Apo2L/TRAIL + obatoclax induced strong immunoreactivity. Bar, 25 μm. Fluorescent signal was quantitated as in A and is presented in a graph below the fluorescence images. **, P < 0.01, compared with untreated cells (ANOVA with Tukey’s least significant difference post-test).
Of interest, Apo2L/TRAIL treatment of cholangiocarcinoma cells is not sufficient to cause apoptosis but instead promotes cell migration and invasion, acting potentially to promote a malignant phenotype (3). This is consistent with alternate signaling pathways activated by Apo2L/TRAIL; indeed, we showed that the increased migration and invasion of cholangiocarcinoma cells was caused by Apo2L/TRAIL-induced nuclear factor-κB activation (3). Nuclear factor-κB can induce apoptosis resistance by transcriptional increases in antiapoptotic Bcl-2 family members. This raises the possibility that obatoclax acts to sensitize cholangiocarcinoma cells to Apo2L/TRAIL by inhibiting a nuclear factor-κB–mediated survival signal, in effect converting the Apo2L/TRAIL effect from prosurvival to apoptogenic; indeed, nuclear factor-κB inhibition sensitizes hepatoma cells to Apo2L/TRAIL killing (39). However, in cholangiocarcinoma cells, this is unlikely to be the operative mechanism of action, as nuclear factor-κB inhibition does not sensitize these cells to Apo2L/TRAIL killing (3).

In summary, our studies suggest that obatoclax reverses Apo2L/TRAIL resistance in cholangiocarcinoma cells. Because human cholangiocarcinomas (compared with cell lines) express Apo2L/TRAIL (3), it is possible that obatoclax might have single-agent antitumor activity in vivo. Accordingly, obatoclax or analogous compounds merit further preclinical investigation and possible evaluation for the treatment of cholangiocarcinoma.

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

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