Inhibition of Bcl-2 anti-apoptotic members by obatoclax potently enhances sorafenib-induced apoptosis in human myeloid leukemia cells through a Bim-dependent process

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Interactions between the multi-kinase inhibitor sorafenib and the BH3-mimetic obatoclax (GX15-070) were examined in human myeloid leukemia (AML) cells. Treatment with sorafenib/obatoclax induced pronounced apoptosis in and reduced the clonogenic growth of multiple AML lines and primary AML cells but not normal CD34+ cells. Sorafenib triggered rapid and pronounced Mcl-1 down-regulation accompanied by enhanced binding of Bim to Bcl-2 and Bcl-xL, effects that were abolished by obatoclax co-administration. Notably, shRNA knockdown of Bim, Bak, or Bax, but not Noxa, significantly attenuated obatoclax/sorafenib lethality, whereas ectopic expression of Mcl-1 exerted a protective effect. Furthermore, exposure of leukemia cells to sorafenib and obatoclax markedly induced autophagy, reflected by rapid and pronounced LC3 processing and LC3-GFP punctate formation. Multiple autophagy inhibitors or VPS34 knockdown, significantly potentiated sorafenib/obatoclax lethality, indicating a cytoprotective role for autophagy in this setting. Finally, studies in a xenograft mouse model revealed that combined sorafenib/obatoclax treatment markedly reduced tumor growth and significantly prolonged survival in association with Mcl-1 downregulation and apoptosis induction, whereas agents administered individually had only modest effects. These findings suggest that combining sorafenib with agents that inhibit Mcl-1 and Bcl-2/-xL such as obatoclax may represent a novel and potentially effective strategy in acute myeloid leukemia.
INTRODUCTION

Members of the Bcl-2 family of apoptotic regulatory proteins are frequently dysregulated in diverse cancers, particularly hematological malignancies such as acute myelogenous leukemia (AML). Such aberrations include over-expression of anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1, as well as decreases/loss of pro-apoptotic members such as Bim, Bax, natural born killer (Nbk)/Bcl-2-interacting killer (Bik). The ultimate consequences of these perturbations are defects in apoptosis that lead to enhanced cell survival as well as increased resistance to various chemotherapeutic drugs. To circumvent such problems, several strategies have been developed which directly target anti-apoptotic Bcl-2 family members. Among these is, obatoclax (GX15-070), a small molecule inhibitor that targets all pro-survival Bcl-2 members including Bcl-2, Bcl-xL, Bcl-W, as well as Mcl-1. Preclinical studies demonstrated that obatoclax exhibits potent anti-tumor activity in various cancer cell types including leukemia. It is currently undergoing phase I and II clinical evaluation. Obatoclax exerts its antitumor activity through multiple mechanisms. For example, it has been shown to trigger apoptosis by dissociating the pro-apoptotic protein Bak from both Mcl-1 and Bcl-xL in conjunction with release of Bim from Mcl-1 and Bcl-2. However, the ability of obatoclax to induce death in Bax/Bak-deficient cells prompted the search for additional mechanisms of lethality. In this context, obatoclax has been reported to induce autophagy- or necroptosis-dependent cell death. Finally, obatoclax may also inhibit cell growth by inducing cell cycle arrest in S-G2 phase.
Sorafenib was originally developed as a C-Raf and B-Raf inhibitor, but was subsequently shown to inhibit multiple other kinases, including FLT3, VEGFR-2, VEGFR-3, PDGFR-β, c-Kit, among others. It is currently approved for the treatment of refractory renal cell and hepatocellular carcinoma. When administered at standard doses (e.g., 400 mg po BID daily), steady state levels in excess of 10 µM have been reported. To date, interest in sorafenib in AML has focused on mutant FLT3 forms of the disease. However, several groups, including our own, have shown that pharmacologically achievable concentrations of sorafenib kill diverse malignant cell types, including wild-type FLT3 human leukemia cells, in association with down-regulation of Mcl-1 protein expression. In human leukemia cells, this stems from a translational inhibitory mechanism. In this setting, Mcl-1 down-regulation has been shown to play a significant functional role in sorafenib lethality.

In addition to the well-established role of Mcl-1 in opposing sorafenib activity, recent evidence suggests that sorafenib lethality may also be attenuated by Bcl-2 and Bcl-xL, raising the possibility that an agent capable of inhibiting all three antiapoptotic proteins (i.e., Mcl-1, Bcl-2, and Bcl-xL) might be particularly effective in potentiating sorafenib anti-leukemic activity. To test this hypothesis, we have examined anti-leukemic interactions between obatoclax and sorafenib in human leukemia cells, focusing on those with wild-type FLT3. Our results indicate that combined treatment with sorafenib and obatoclax exhibits potent anti-leukemic activity in vitro and in vivo, and suggest that this strategy warrants further investigation.
METHODS

Cells. Human leukemia U937, HL-60, and MV4-11 cells were cultured as previously reported. U937 cells stably overexpressing wild-type Mcl-1 or Bim constructs were previously described. U937 cells stably expressing short hairpin RNA (shRNA) directed against Bax, Bak, or Noxa were generated as previously described. Knockdown of Bim was accomplished by transfecting U937 cells with two distinct microRNA-adapted shRNA constructs specifically designed against human Bim (shBim#1: cat# RHS1764-9687395, and shBim#2: cat# RHS1764-9687431; Open Biosystems, Huntsville, AL). U937 cells transfected with shRNA constructs against GFP (shGFP) were used as a control for various shRNA expressing cells. To knockdown VPS34, lentiviral particles carrying a pKL01 shRNA construct (cat# RHS3979-9605204; Open Biosystems) were generated using a Lenti-X HTX packaging system (Clontech) and transduced into U937 cells. Cells were selected in the presence of puromycin for one week and monitored for VPS34 expression level. U937 cells infected with lentiviruses carrying scrambled sequence constructs were used as negative controls.

Isolation of patient-derived leukemic blasts. Leukemic blasts were obtained from the bone marrows of patients with acute myeloblastic leukemia (AML), FAB subtype M2. These studies have been sanctioned by the Investigational Review Board of Virginia Commonwealth University/Medical College of Virginia, and all patients provided informed consent. In each case, the percentage of blasts in the peripheral blood was > 70%. Bone marrow was collected, and mononuclear cells isolated as previously described.
**FLT3 mutations analysis.** FLT3 mutations analysis was performed on genomic DNA extracted from primary bone marrow blasts isolated from patients with AML as previously described\textsuperscript{25}.

**Isolation of CD34\textsuperscript{+} cells.** Normal bone marrow CD34\textsuperscript{+} cells were obtained with informed consent from patients undergoing routine diagnostic procedures for non-myeloid hematopoietic disorders. CD34\textsuperscript{+} cells were isolated from mononuclear cell preparations as previously described.\textsuperscript{28}

**In vivo studies**

Animal studies were conducted under an approved protocol by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Female athymic nude mice were purchased from Charles River laboratories, and were inoculated subcutaneously with 2.5 x 10\textsuperscript{6} parental or luciferase-expressing U937 cells. Mice were monitored for tumor growth visually or using the IVIS 200 imaging system (Xenogen Corporation, Alameda, CA). Once tumors became apparent, the mice were separated in 4 groups of 5 mice each and treated every 24 h for six days a week with sorafenib (80 mg/kg) administered by gavage, obatoclax (3.5 mg/kg) by intramuscular (IM) injection, or the combination of sorafenib and obatoclax. Control mice were treated with equal volumes of vehicle alone. Measurement of animal body weights were performed twice a week throughout the study as an indicator of toxicity. Tumor volumes were calculated using the formula (L x W\textsuperscript{2})/2, with L and W representing length and width respectively, and when tumor size reached 2000 mm\textsuperscript{3}, mice were euthanized.
Reagents. Sorafenib was provided by Bayer Pharmaceuticals Corporation (West Haven, CT) and the National Cancer Institute, NIH (Bethesda, MD). Obatoclax was provided by GeminX, Biotechnologies, Inc. and the National Cancer Institute. HA14-1, gossypol, 3-methyladenine, chloroquine, and bafilomycin A1 were purchased from Sigma Aldrich (St. Louis, MO). All reagents were formulated as recommended by their suppliers.

Assessment of apoptosis. The extent of cell death was routinely assessed by 7-AAD staining assay as previously described. Parallel studies employing annexin V/PI analysis yielded essentially equivalent results.

Autophagy

EGFP-LC3 fusion cDNA was cut from pEGFP-C2 vector, a gift from Dr. Finkel T (Addgene #24920, Cambridge MA) and sub-cloned into PLVX-puro lentiviral vector (Clontech). Lentiviral particles were generated as above and used to infect U937 and MV4-11 cells. Cells expressing EGFP-LC3 were sorted by FACS before treatment. After exposure to the designated agents, cells were fixed with 4% paraformaldehyde, mounted onto slides in mounting medium containing DAPI (SouthernBiotech, Birmingham, AL), and analyzed for autophagy using a Zeiss LSM 510 confocal microscope.

Fusion between autophagosomes and lysosomes was assessed by co-localization of LC3 (autophagosome marker) and LAMP1 (lysosome marker) using confocal microscopy. Briefly, cells expressing EGFP-LC3 were fixed as above and permeabilized with 0.25% Triton X-100. LAMP1 was immuno-detected with anti-LAMP1 antibodies (H5G11; Santa Cruz Biotechnology, Santa Cruz, CA) using Alexa-Fluor-647-conjugated secondary antibodies.
LC3 was detected with GFP fluorescence amplified by Alexa Fluor 488-conjugated anti-GFP antibodies (Molecular Probes).

**Immunoprecipitation and immunoblotting.** Cells were lysed in CHAPS buffer after which 500 μg of protein lysates were subjected to immunoprecipitation using designated antibodies. Immunoblotting was performed using the immunoprecipitates or the whole cell lysates as previously described in detail. Primary antibodies were: Polyclonal Bax, Bcl-2, and Mcl-1 (PharMingen; San Diego, CA). Noxa (Alexis Corp; San Diego, CA). Poly(ADP-ribose) Polymerase (PARP) (Biomol Research Laboratories, Plymouth Meeting, PA). Cleaved caspase-9, and cleaved caspase-3, ERK1/2, and ATG5 (Cell Signaling Technology; Beverly, MA). AIF, cytochrome c, polyclonal Bak, Bcl-xL, SQSTM1/p62, VPS34, and beclin-1, (Santa Cruz Biotechnology). LC-3 (Novagen). Bim, and α-tubulin (Calbiochem).

**Bax and Bak conformational change.** Cells were lysed in CHAPS buffer and protein lysates subjected to immunoprecipitation using anti-Bax 6A7 (Sigma) or anti-Bak Ab-1 antibodies (Calbiochem) that recognize only conformationally changed Bax or Bak protein. Immunoprecipitates were then subjected to immunoblotting analysis using anti-Bax or anti-Bak polyclonal antibodies.

**Subcellular fractionation.** Cytosolic and membrane fractions were separated as previously described.

**Statistical analysis.** The significance of differences between experimental conditions was determined using the Student’s t test for unpaired observations. Synergistic interactions were evaluated using Median Dose Effect analysis employing the Calcusyn software program.
Survival of mice following treatment was evaluated by Kaplan-Meier analysis.

RESULTS

Combined exposure of human leukemia cells to sorafenib and obatoclax results in the pronounced induction of cell death in association with profound mitochondrial injury and caspase activation.

To examine interactions between sorafenib and obatoclax, U937 cells were exposed to various concentrations of these agents alone or in combination for 48 h, after which the extent of cell death was monitored by the 7-AAD assay. While 1.5 μM obatoclax was only minimally toxic by itself, it substantially increased cell death of pharmacologically achievable concentrations of sorafenib (e.g., 4 - 8 μM) (Figure 1A). Analogously, the lethality of marginally toxic obatoclax concentrations (e.g., 0.5 - 2 μM) was significantly increased by co-exposure to 7.5 μM sorafenib (Figure 1B).

Time course analysis of cells exposed simultaneously to 7.5 μM sorafenib and 1.5 μM obatoclax revealed approximately 30 % cell death at 24 h, and more pronounced lethality after 48-72 h (60-80 %, Figure 1C). Similar results were obtained with Wright-Giemsa staining or trypan blue assays (data not shown). Median Dose Effect analysis of cells exposed to sorafenib and obatoclax for 48 h at a fixed ratio yielded Combination Index values considerably less than 1.0, indicating a highly synergistic interaction (Figure 1D). Enhanced lethality following combined sorafenib/obatoclax exposure was also observed in other leukemia cell lines including the FLT3-ITD-dependent MV4-11, and promyelocytic leukemia HL-60 cells (Figure 1E).
Notably, in MV4-11 cells, lower sorafenib concentrations were required (e.g., 75 nM), presumably due to inhibitory effects of sorafenib on FLT3. Combined exposure to sorafenib and obatoclax was associated with profound mitochondrial damage, reflected by the pronounced release of cytochrome c and AIF into the cytosol in U937 cells (Figure 1F), the marked cleavage/activation of caspases-3, and -9, and PARP in U937 as well as MV4-11 and HL-60 cells (Figure 1G), and the pronounced loss in mitochondrial membrane potential (Δψ, supplemental Figure 1A). In sharp contrast, individual exposure of cells to sorafenib or obatoclax had only minimal effects. Notably, pre-treatment of cells with the pan-caspase inhibitor Q-VD-OPH sharply diminished sorafenib/obatoclax-mediated lethality (Supplemental Figure 1B). Combined treatment also markedly reduced cell growth and viability (Supplemental Figure 1C). Finally, combined exposure to sorafenib and very low concentrations of obatoclax (75 nM) resulted in a marked decline in U937 cell colony formation, whereas the agents administered alone exerted only modest effects (Figure 1H).

Parallel studies using the annexin V/PI assay revealed that co-exposure of human leukemia cells to sorafenib and HA14-1, another BH3-mimetic Bcl-2 antagonist for 24 h also resulted in the marked induction of apoptosis (Supplemental Figure 2A), in association with the striking release of cytochrome c and AIF into the cytosol (Supplemental Figure 2B) and cleavage of caspase-3, caspase-9 and PARP (Supplemental Figure 2C). In addition, Median Dose Effect analysis performed in U937 cells revealed highly synergistic interactions between sorafenib and HA14-1 (Supplemental Figure 2D) analogous to results obtained with sorafenib and obatoclax. Lastly, sorafenib also enhanced the lethality of the BH3-mimetic gossypol in U937 cells (Supplemental Figure 2E).
Co-treatment with sorafenib and obatoclax increases lethality in primary AML blasts while largely sparing normal CD34+ cells.

Parallel studies using the 7-AAD assay revealed that simultaneous administration of sorafenib and obatoclax resulted in a significant increase in cell death in primary leukemic blasts isolated from 4 patients with AML (FAB classification M2; 3 patients with wild-type FLT3 and 1 patient with a FLT3-ITD mutation) (Figure 2A). Similar results were obtained when Wright Giemsa-stained cells were evaluated (data not shown). Further analysis performed on blasts isolated from patient #1 showed enhanced cleavage of caspase-3 and PARP in cells co-treated with sorafenib and obatoclax compared to cells treated with the agents alone (Figure 2B). Interestingly, exposure to comparable or significantly higher concentrations of sorafenib and obatoclax alone or in combination exhibited minimal lethality toward normal CD34+ cells (Figure 2C). Furthermore, the clonogenic potential of primary AML specimens was markedly diminished by combined treatment with sorafenib and obatoclax (Figure 2D), whereas the clonogenicity of normal CD34+ cells was largely unaffected (Figure 2E).

Sorafenib/obatoclax-mediated lethality involves Bax and Bak

In view of the central roles that the multi-domain proteins Bax and Bak play in apoptosis, activation of these proteins was examined. While treatment with sorafenib (7.5 μM) or obatoclax (1.5 μM) alone minimally induced Bak and Bax conformational change, effects of combined treatment were very pronounced (Figure 3A and 3B). In contrast, no major changes in Bax expression was observed in cells treated with obatoclax alone or in combination with sorafenib. On the other hand, a modest decrease in Bak protein level was observed following obatoclax treatment. To test the functional role of Bax and Bak in sorafenib/obatoclax lethality,
U937 cells in which Bax or Bak were knocked down (Figure 3C) were employed. Dose-response studies revealed that knockdown of Bax or Bak rendered cells more resistant to either sorafenib or obatoclax alone (Supplemental Figures 3A and 3B). In addition, these cells displayed significant resistance to sorafenib/obatoclax-mediated caspase activation and cell death (Figures 3C and 3D). These findings indicate that Bax and Bak play significant functional roles in the anti-leukemic activity of the sorafenib/obatoclax regimen.

**Mcl-1 downregulation is required for sorafenib/obatoclax-mediated apoptosis.**

Previously, we and others reported that sorafenib diminishes Mcl-1 protein expression in human leukemia cells by inhibiting translation through a MEK1/2/ERK1/2 signaling-independent mechanism, and that this phenomenon plays a significant functional role in sorafenib lethality.\(^{16;17;20}\) Consequently, Mcl-1 expression and function was examined in relation to sorafenib and obatoclax responses. Consistent with our previous report,\(^{16}\) Western blot analysis revealed that sorafenib significantly reduced Mcl-1 protein levels. Interestingly, obatoclax induced similar effects, and combined treatment (24 h) essentially abrogated Mcl-1 expression (Figure 4A). Comparable results were obtained in HL-60 and MV4-11 cells as well as primary AML blasts (Figure 4B). In contrast, no major changes were noted in the levels of other anti-apoptotic family members i.e., Bcl-2 or Bcl-xL (Figure 4A). In addition, very modest down-regulation of Bim protein levels was observed following exposure to agents alone or in combination. However, in marked contrast, and consistent with a previous report,\(^{33}\) Noxa was markedly down-regulated by sorafenib (Figure 4A). However, this effect was largely prevented by co-treatment with obatoclax, which by itself modestly increased Noxa protein levels at 6 h, but not at 24 h.
To assess the functional significance of Mcl-1 down-regulation in sorafenib/obatoclax-induced lethality, U937 cells ectopically expressing Mcl-1 were employed. As anticipated, these cells were significantly more resistant than control cells to sorafenib (Supplemental Figure 3C). Interestingly, these cells were also significantly more resistant to obatoclax than control cells, although the degree of resistance was less than that observed in the case of sorafenib (Supplemental Figure 3D). Notably, combined treatment with 7.5 μM sorafenib and 1.5 μM obatoclax, which resulted in pronounced cell death in empty-vector cells, only modestly increased cell death in Mcl-1-overexpressing cells (Figure 4C). However, Mcl-1-mediated resistance was fully reversed by increasing agent concentrations (i.e., 12 μM sorafenib, and 3 μM obatoclax, Figure 4C), accompanied by sharply increased caspase-3 cleavage/activation, and PARP cleavage (Figure 4D). Together, these findings argue that Mcl-1 down-regulation in cells exposed to sorafenib and obatoclax plays a significant functional role in the lethality of this regimen.

**Bim, but not Noxa, plays a critical functional role in anti-leukemic activity of sorafenib/obatoclax.**

Previously, we and others have reported that the BH3-only protein Bim (Bcl-2-interacting mediator of cell death) plays an important functional role in sorafenib-mediated apoptosis. Therefore, the hypothesis that sorafenib/obatoclax might enhance Bim activation by interfering with Bcl-2, Bcl-xL, and Mcl-1 interactions was examined. Interestingly, immunoprecipitation experiments revealed that sorafenib alone significantly increased Bim binding to Bcl-2 (Figure 4E; upper panel) and Bcl-xL (Figure 4E; lower panel), presumably due to release of Bim from
Mcl-1 following down-regulation of the latter. Significantly, these effects were essentially abrogated by co-treatment with obatoclax. To test the functional role of Bim in sorafenib/obatoclax-mediated lethality, two shRNA constructs (shBim#1, shBim#2) designed against Bim were separately transfected into U937 cells, and stable clones exhibiting significant Bim knockdown with each construct were employed (Figure 5A). Bim knockdown cells were significantly more resistant to sorafenib/obatoclax-mediated lethality than their control counterparts, reflected by diminished cleavage of caspase-3 and its downstream substrate PARP (Figure 5A), cytochrome c release into the cytosol (Figure 5B), and 7-AAD uptake (Figure 5C). Knockdown of Bim in MV4-11 cells also significantly diminished sorafenib/obatoclax lethality (Supplemental Figures 4A and 4B). Furthermore, cells ectopically expressing Bim displayed significantly enhanced sensitivity to the sorafenib/obatoclax regimen compared to control cells transfected with a pcDNA3.1 construct as reflected by increased 7-AAD positivity (Figure 5D), and enhanced cleavage of caspase-3 and PARP (Supplemental Figure 4 C). Together, these findings suggest that Bim plays an important functional role in sorafenib/obatoclax-mediated lethality.

Finally, previous studies indicated that the pro-apoptotic protein Noxa may also contribute to cell death induced by treatment involving obatoclax or sorafenib in some settings. To determine whether Noxa played a functional role in sorafenib/obatoclax-mediated cell death, U937 cells in which Noxa protein was knocked down with shRNA were employed. In contrast to results involving Bim, a 7-AAD staining assay revealed that U937 cells in which Noxa expression was markedly decreased (Figure 5F) remained fully sensitive to sorafenib/obatoclax lethality (Figure 5E). Consistent with this finding, no major changes were
observed in caspase-3 or PARP cleavage (Figure 5F), arguing against the possibility that Noxa plays a major functional role in sorafenib/obatoclax-mediated apoptosis in human leukemia cells.

**Autophagy plays a protective role against sorafenib/obatoclax lethality.**

Because both sorafenib and obatoclax have been implicated in induction of autophagy in diverse systems,\textsuperscript{10,11} we examined whether this process might play a role in sorafenib/obatoclax antileukemic activity. As shown in Figure 6A, obatoclax elicited a rapid and robust induction of LC3 processing in U937 cells, which occurred as early as 2 h after treatment and persisted over the ensuing 20 h post-treatment. This effect was also observed with combined exposure to obatoclax and sorafenib, although it was slightly less pronounced. However, sorafenib alone had no discernible effect on LC3 processing. Similar results were obtained in HL-60 cells (Supplemental Figure 5F). In contrast, no major changes were observed in the expression of beclin-1 (BCN1) or ATG5 protein levels with any treatments. Consistent with LC-3 processing, confocal microscopic analysis of U937 cells expressing an LC3-GFP fusion protein revealed a marked increase in autophagosomes, as indicated by enhanced LC3-GFP punctate formation/aggregation in cells exposed for 6 h to obatoclax alone or in combination with sorafenib (but not to sorafenib alone) (Figure 6B). Similar results were observed in MV4-11 cells (Supplemental Figure 5A). Furthermore, immunofluorescence studies using LC3-EGFP (an autophagosome marker) and LAMP1 (a lysosome marker) revealed marker co-localization after treatment with obatoclax alone or in combination with sorafenib, indicating fusion between autophagosomes and lysosomes (Figure 6C and Supplemental Figure 5B). Furthermore p62/SQSTM1, a marker of autophagy flux, significantly decreased following treatment (24 h) with obatoclax alone or obatoclax/sorafenib (Supplemental Figure 5C).
Interestingly interference with autophagy using a series of autophagy inhibitors (e.g., 3MA, chloroquine, or bafilomycin A1) strikingly potentiated sorafenib/obatoclax lethality (Figure 6D). In contrast, these agents did not result in significant increases in the lethality of obatoclax or sorafenib alone (Supplemental Figure 5D). Western blot analysis (Figure 6E) demonstrated diminished LC3 processing by sorafenib/obatoclax in the presence of 3MA, indicating inhibition of autophagy, accompanied by enhanced apoptosis, reflected by increased cleavage of PARP. Similar findings were observed in HL-60 cells (Supplemental Figures 5E and 5F). Chloroquine however, which inhibits late-stage autophagy by blocking lysosomal acidification, did not decrease LC3 processing (Supplemental Figure 5G), as previously reported.37,38 In accord with these findings, knock down of VPS34, an essential protein for autophagy 37 significantly enhanced obatoclax/sorafenib lethality (Figure 6F). Finally, consistent with a cytoprotective role for autophagy, Bim knockdown-mediated resistance to obatoclax/sorafenib lethality was associated with increased LC3 processing (Supplemental Figure 6). Together these findings argue that autophagy primarily plays a protective role against the lethality of this regimen.

**Combined treatment with sorafenib and obatoclax results in apoptosis induction, tumor growth reduction, and enhanced survival in an in vivo leukemia xenograft model.**

To determine whether co-treatment with sorafenib and obatoclax exhibits anti-leukemic activity in vivo, nude mouse xenografts inoculated with U937 cells were employed. Notably, sorafenib (80 mg/kg) administered alone resulted in a clear inhibition of tumor growth (Figure 7A, 7B), and this effect was significantly enhanced by combined treatment with 3.5 mg/kg obatoclax, which by itself exhibited only modest effects (Figure 7A and 7B). Significantly, TUNEL assays
and Western blot analysis performed on tumor tissue excised from animals treated with two
doses of each agent alone or in combination over a 24 h interval revealed that combined, but not
individual, treatment induced apoptosis, reflected by a clear increase in TUNEL positivity
(Figure 7C), and sharply increased caspase-3 processing and PARP cleavage in association with
a marked decline in Mcl-1 levels (Figure 7D). After 8 days of treatment, Mcl-1 levels in tumors
were significantly down-regulated by either agents alone or in combination (data not shown).
These events correlated with significantly prolonged survival of tumor-bearing mice treated with
sorafenib/obatoclax and, to a lesser extent, with sorafenib alone (Figure 7E). In contrast,
obatoclax administered alone did not prolong survival. These findings were confirmed in studies
of mice inoculated with luciferase-expressing U937 cells (Supplemental Figure 7A). Finally,
treatment with agents alone or in combination did not lead to major changes in mouse weights or
in white or red blood cell counts (Supplemental Figures 7B and 7C respectively), nor were
changes in behavior or hair loss observed. Together, these findings indicate that combined
treatment with sorafenib and obatoclax significantly reduces tumor growth in association with,
Mcl-1 down-regulation, and prolongs survival in leukemia-bearing mice.
DISCUSSION

Members of the Bcl-2 family of apoptotic regulatory proteins are frequently dysregulated in various transformed cells, including those of hematopoietic origin. In previous reports, we and others have demonstrated that the multikinase inhibitor sorafenib potently induces apoptosis in diverse cancer cell types, including leukemia, through a process involving Mcl-1 down-regulation. Interestingly, Mcl-1 down-regulation by sorafenib was found to be related to translation inhibition through a mechanism(s) independent of MEK1/2/ERK1/2 signaling. Recent evidence suggests that Bcl-2 and Bcl-xL may also confer resistance to sorafenib-mediated apoptosis in transformed cells raising the possibility that inhibition of the anti-apoptotic proteins Bcl-2 and Bcl-xL may enhance sorafenib lethality. The present results indicate that combined sorafenib/obatoclax exposure triggers pronounced lethality in and markedly reduces the clonogenic survival of various primary AML blast specimens and cell lines, while exerting only modest effects on normal CD34+ cells. Importantly, in vivo studies demonstrate that combined sorafenib/obatoclax treatment markedly inhibits leukemia growth and significantly prolongs survival in association with diminished leukemia cell Mcl-1 down-regulation, and caspase activation, thus recapitulating effects occurring in vitro.

The observed Mcl-1 downregulation by either sorafenib or obatoclax is in accord with previous results from our and other groups. While inhibition of MEK1/2/ERK1/2 has been shown to diminish Mcl-1 expression, sorafenib-mediated Mcl-1 down-regulation appears to proceed through a translational mechanism that does not depend upon ERK1/2 inactivation. In contrast, relatively little is known about the mechanism(s) by which obatoclax down-regulates Mcl-1. Given evidence that obatoclax up-regulates Noxa expression in
some cell types,\textsuperscript{35,36} it is possible that obatoclax may induce Noxa, which enhances Mcl-1 protein degradation.\textsuperscript{41} However, the observation that obatoclax diminished Mcl-1 levels in the absence of Noxa up-regulation (e.g., in UPN-1 or Jeko mantle cell lymphoma lines)\textsuperscript{35} argues against a primary role for Noxa in this phenomenon, at least in some cell types. Regardless of the precise mechanism(s) by which Mcl-1 down-regulation occurred, the finding that ectopic Mcl-1 expression significantly attenuated sorafenib/obatoclax-induced lethality suggests that Mcl-1 down-regulation contributes functionally to cell death in this setting.

It is well established that Mcl-1 plays an important role in leukemia survival.\textsuperscript{42} However, it is unlikely that Mcl-1 down-regulation in isolation can trigger pronounced cell death.\textsuperscript{43} Instead, it is more likely that Mcl-1 downregulation cooperates with other events (e.g.; MEK/ERK inactivation, downregulation of cFLIPL, cIAP2, and survivin among others\textsuperscript{19,21,44}) to lower the apoptotic threshold. In support of this notion, obatoclax, which inhibits Mcl-1 as well as Bcl-2/Bcl-xL,\textsuperscript{4} has been shown to induce apoptosis administered alone in human leukemia cells, but at considerably higher concentrations than those employed here.\textsuperscript{5} Despite the observed cytoprotection conferred by ectopic expression of Mcl-1, it is noteworthy that increasing concentrations of sorafenib and obatoclax (approximately two-fold) restored apoptosis to levels equivalent to those observed with lower drug concentrations in empty-vector control cells. Such findings raise the possibility that this strategy may be effective against some leukemia cells exhibiting increased Mcl-1 expression.

It is currently thought that Mcl-1 anti-apoptotic actions primarily involve interactions with the pro-apoptotic proteins Bak, and Bim.\textsuperscript{45} Of note, the BH3-only protein Bim binds with
equivalent avidity to the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. In this context, treatment with sorafenib increased binding of Bim to Bcl-2 and Bcl-xL, presumably a consequence of Mcl-1 down-regulation accompanied by release of Bim, and that this effect was essentially abrogated by obatoclax co-treatment. While obatoclax has been shown to interfere with Bim binding to Bcl-2, its capacity to inhibit the Bim-Bcl-xL association has not, to the best of our knowledge, previously been described. Together, these observations raise the possibility that combined treatment increases free Bim through two mechanisms: a) reduction in Mcl-1 expression, thus freeing Bim; and b) release of Bim from Bcl-2/Bcl-xL, to which it might otherwise bind following Mcl-1 down-regulation. The consequences of these events are activation of Bax/Bak and resulting induction of cell death. In addition to down-regulation of Mcl-1, obatoclax also has been shown to untether Bak from Mcl-1 and to promote Bak activation. Together these findings support the notion that combined treatment leads to release of Bim from all three anti-apoptotic proteins (i.e., Bcl-2, Bcl-xL, and Mcl-1), leading to Bak and Bax activation and apoptosis. This interpretation is supported by the observations that knockdown of Bax, Bak, or particularly Bim with shRNA, or ectopic expression of Mcl-1, significantly diminished sorafenib/obatoclax-mediated lethality.

Recent studies have shown that Noxa plays an important role in cell death mediated by obatoclax when this agent is combined with bortezomib in mantle cell lymphoma cells or with tunicamycin in melanoma cells. In the latter, but not the former, report, modest up-regulation of Noxa was observed. In the present studies, while sorafenib sharply down-regulated Noxa protein levels as previously described, obatoclax, either alone or in combination with sorafenib, increased Noxa expression in accord with previous results. However such increases were
modest and transient, appearing at 6 h but not at 24 h. Significantly, knockdown of Noxa with shRNA failed to diminish obatoclax/sorafenib lethality, arguing that Noxa up-regulation does not play a critical functional role in this setting. This may be explained by evidence that the primary mechanism by which Noxa promotes apoptosis involves neutralization/down-regulation of Mcl-1,\textsuperscript{41,47} which under the present circumstances was largely eliminated through the actions of sorafenib.

Of note, obatoclax increased the activity of lower sorafenib concentrations to induce cell death in FLT3-mutated cells, which are known to be highly susceptible to the latter agent both \textit{in vitro} and \textit{in vivo}.\textsuperscript{14,15} In such cells, interactions between obatoclax and lower concentrations of sorafenib may involve mechanisms other than or in addition to translational inhibition of Mcl-1 (e.g., potentiation of the lethal consequences of sorafenib-mediated FLT3-ITD inhibition by obatoclax). Separate studies designed to test this concept are currently in progress.

It is interesting that obatoclax administered either alone or in combination with sorafenib, induced a marked increase in autophagic markers, including induction of LC3-II and formation of GFP-LC3 punctate/aggregates in human leukemia cells. Notably, LC3 processing was slightly reduced in cells exposed to combined treatment compared to obatoclax alone. However, the pronounced increase in LC3-GFP punctuate formation and fusion between autophagosomes and lysosomes observed with combined treatment was similar to that seen with obatoclax alone. Autophagy represents a versatile and dynamic cellular response to diverse noxious stimuli that, in most cases protects cells, but can also, under some circumstances, contribute to their demise.\textsuperscript{48} It should be noted that increases in autophagic markers such as processed LC3 or the formation
of LC3-GFP punctuate do not necessarily reflect increased autophagic flux, but can also represent inhibition of autophagosome maturation and lysosomal degradation. In this regard, LC3 processing can also be initiated by certain ill-defined autophagy-independent mechanisms. However, previous findings implicating obatoclax in the induction of autophagy, evidence of increased fusion between autophagosomes and lysosomes, and the observations that diverse autophagy inhibitors (e.g. 3MA, chloroquine, bafilomycin A1) or knockdown of VPS34 markedly potentiated sorafenib/obatoclax-mediated lethality, argue for a protective role for autophagy induction in the current setting. Finally, these findings raise the possibility that clinically relevant autophagy inhibitors (e.g., chloroquine) may further enhance sorafenib/obatoclax anti-leukemic activity.

In addition to marked in vitro interactions, the sorafenib/obatoclax regimen elicited a pronounced reduction in tumor growth in a leukemia xenograft murine model accompanied by a significant increase in survival. Importantly, these events were also associated with Mcl-1 down-regulation and enhanced apoptosis in leukemia cells exposed to these agents in vivo. They also suggest that sufficiently high sorafenib and obatoclax concentrations can be achieved in vivo to recapitulate at least some of the actions observed in vitro e.g. Mcl-1 down-regulation. Interestingly, little toxicity was observed in animals with the doses and schedules used in these studies. Previous studies have described the development of neurotoxicity when obatoclax was administered intravenously to nude mice. We observed similar phenomena (data not shown), but this problem was largely resolved by employing intramuscular injections. Although the ability of this regimen to eradicate leukemia stem cells remains to be determined, the present in vivo findings, along with in vitro evidence that the sorafenib/obatoclax regimen is active against at
least some primary AML cells and exhibits minimal toxicity toward normal CD34⁺ cells, suggest that this strategy warrants further consideration in AML. Accordingly, plans to evaluate the tolerability of this regimen through a phase I trial in patients with refractory AML, including patients with either FLT3-ITD-mutated or –wild-type disease, are currently underway.
ACKNOWLEDGMENTS

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AUTHORSHIP

M. Rahmani designed and performed the research, analyzed data and wrote the manuscript. M. Mayo and E. Attkisson performed the research. A. Fereirra-Gonzalez and D. Williams analyzed data. S. Grant designed research, identified patient samples and wrote the manuscript.

CONFLICT OF INTEREST DISCLOSURE:

The authors have no conflict of interest to disclose.
REFERENCE LIST


40. Domina AM, Vrana JA, Gregory MA, Hann SR, Craig RW. MCL1 is phosphorylated in the PEST region and stabilized upon ERK activation in viable cells, and at additional sites with cytotoxic okadaic acid or taxol. *Oncogene*. 2004;23 (31) :5301-5315.


FIGURE LEGENDS

Figure 1. Combined treatment with sorafenib and obatoclax results in a marked induction of cell death in association with profound mitochondrial injury and caspase activation, and diminishes the colony-formation capacity of human leukemia cells.

(A) U937 cells were exposed to the designated concentrations of sorafenib alone (empty circles) or in combination with 1.5 μM obatoclax (Ob, filled circles) for 48 h after which the percentage of apoptotic cells was determined by the 7-AAD staining assay. * = significantly greater than sorafenib alone; P < 0.05; ** = P < 0.01. (B) U937 cells were exposed to the designated concentrations of obatoclax alone (empty circles) or in combination with 7.5 μM sorafenib (filled circles), for 48 h after which cell death was determined as above. * = significantly greater than obatoclax alone; P < 0.02; ** = P < 0.01. (C) Cells were exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) alone or in combination for the indicated intervals, after which the percentage of dead cells was determined as above. * = significantly greater than either agent alone; P < 0.05; ** = P < 0.002. (D) Median Dose Effect analysis of cell death induction by sorafenib and obatoclax. U937 cells were exposed to varying concentrations of obatoclax and sorafenib at a fixed ratio (1:5), for 48 h after which extent of cell death was monitored with the 7-AAD staining assay. Combination Index (C.I.) values were determined in relation to the Fractional Effect using a commercially available software program as described in Methods. C.I. values < 1.0 correspond to a synergistic interaction. (E) MV4-11 and HL-60 cells were exposed to sorafenib (75 nM and 7.5 μM respectively) and obatoclax (0.5 μM and 2 μM respectively), either individually or in combination for 48 h, after which the percentage of dead cells was determined by the 7-AAD assay. * = significantly greater than values for either agent alone; P < 0.02. (F) U937 cells were exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) alone or in
combination for 24 h after which mitochondria-free cytosolic fractions were obtained as described in Methods, and subjected to Western blot analysis to monitor the release of cytochrome c and AIF into the cytosol. For this and all subsequent Western blot analysis, blots were subsequently re-probed with anti-tubulin (Tub) antibodies to document equivalent loading and transfer, and the blots shown are representative of at least 3 separate experiments. (G) U937, MV4-11 and HL-60 cells were exposed to sorafenib and obatoclax individually or in combination as in E and F for 48 h after which protein lysates were prepared and subjected to Western blot analysis using the designated antibodies. (H) U937 cells were plated in methylcellulose in the presence of sorafenib (7.5 μM) and obatoclax (75 nM) alone or in combination for 10 days after which colony-forming units (CFUs) were enumerated and expressed as a % of untreated cells. * = significantly less than values for either agent alone; P < 0.02. For A, B, C, E, and H, values represent the means ± SD for 3 separate experiments in which each sample was analyzed in triplicate.

Figure 2. Combined exposure to sorafenib and obatoclax results in enhanced lethality in primary AML cells. (A) Leukemic blasts were isolated as described in Methods from the bone marrow of 4 patients with AML (FAB classification M2; AML#1, AML#2, and AML#4 with wild-type FLT3, and AML#3 with a FLT3-ITD mutation), exposed to sorafenib (7.5 μM) and obatoclax (0.5 μM) for 48 h, after which the extent of cell death was assessed using the 7-AAD analysis. Results are presented as percentage of dead cells specific for each treatment using the formula ((treatment - control)/(100 - control))*100. Cell death for untreated control samples ranged from 10-25%. (B) Alternatively, protein lysates were prepared from AML patient #1 and subjected to Western blot analysis. Densitometric analysis of cleaved PARP and caspase-3 bands
was performed using Adobe Photoshop. Values shown were normalized to ERK1/2 and represent relative changes compared to control. (C) Normal CD34+ cells were isolated as described in Methods from the bone marrow of normal subjects (non-leukemic; N #1, N #2, and N #3) and exposed to increasing concentrations of sorafenib and obatoclax alone or in combination for 48 h, after which the extent of cell death was determined using the 7-AAD analysis. (D) Two primary AML specimens were plated in methylcellulose in the presence of 5 μM sorafenib and 75 nM obatoclax alone or in combination for 14 days, after which colony-forming units (CFUs) were enumerated and expressed as a percentage relative to untreated cells. (E) Normal CD34+ cells from 2 subjects were plated in methylcellulose in the presence of increasing concentrations of sorafenib and obatoclax alone or in combination for 8 days, after which colony-forming units (CFUs) were enumerated and expressed as in (D). For (A, C-E) data for each patient were obtained from a single experiment performed in triplicate; values represent the means ± SD.

**Figure 3. Exposure to sorafenib/obatoclax results in Bak and Bax conformational change while knockdown of these molecules markedly attenuates cell death.** U937 cells were exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) alone or in combination for 30 h after which cells were lysed in buffer containing 1% CHAPS; conformationally changed Bax (A) and Bak (B) proteins were immunoprecipitated using anti-Bax 6A7 and anti-Bak Ab1 antibodies respectively and subjected to Western blot analysis using polyclonal Bax or Bak antibodies. Input lysates were also subjected to Western blot analysis to monitor Bax and Bak protein levels. (C) U937 cells stably transfected with shRNA against GFP (shGFP), Bax (shBax), or Bak (shBak) were treated with sorafenib (7.5 μM) and obatoclax (1.5 μM), for 48 h after which
protein lysates were prepared and subjected to Western blot analysis. Alternatively, the extent of cell death was determined using the 7-AAD staining assay (D). Values represent the means for 3 separate experiments ± S.D. * = significantly lower than values obtained for shGFP cells (P < 0.01).

**Figure 4. Sorafenib/obatoclax-mediated lethality involves Mcl-1 down-regulation.** (A) U937 cells were exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) alone or in combination for 6 or 24 h after which protein lysates were prepared and subjected to Western blot analysis using the designated antibodies. (B) HL-60, MV4-11 cells, and primary blasts were treated with sorafenib (7.5 μM for HL-60 cells and primary blasts; 75 nM for MV4-11 cells) and obatoclax (2 μM for HL-60, and 0.5 μM for MV4-11 and primary blasts) for 28 h after which cells were lysed and protein lysates were subjected to Western blot analysis. (C) U937 cells ectopically expressing Mcl-1 or their empty vector control cells (pCEP4) were treated with the designated concentrations of sorafenib and obatoclax alone or in combination for 48 h after which the extent of cell death was determined using the 7-AAD staining assay. Values represent the means for 3 separate experiments ± S.D. * = significantly less than values for pCEP4 control cells; P < 0.05. ** = P < 0.01. Alternatively, cleavage of PARP and caspase-3 in U937/Mcl-1 cells exposed to 12 μM sorafenib and 3 μM obatoclax was monitored by Western blot analysis (D). (E) U937 cells were treated with sorafenib (7.5 μM) and obatoclax (1.5 μM) individually or together for 8 or 24 h after which cells were lysed and subjected to immunoprecipitation using Bim antibodies. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with either Bcl-2 (upper panel) or Bcl-xL (lower panel) antibodies.
Figure 5. Knockdown of Bim, but not Noxa, significantly diminishes sorafenib/obatoclax-mediated cell death. (A) U937 cells were transfected with 2 shRNA constructs designed against Bim (shBim#1 and shBim#2), and one clone from each transfection was selected. These and shGFP control cells were exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) for 48 h after which Western blot analysis was performed. (B) shBim#1 and shBim#2 cells were treated with sorafenib and obatoclax as above for 6 h after which the cytosolic fraction was isolated and subjected to Western blot analysis. (C) shBim#1, shBim#2, and shGFP cells were exposed to sorafenib and obatoclax as in (A) for 48 h after which the extent of cell death was monitored by the 7-AAD staining assay. * = significantly less than values for shGFP control cells; P < 0.02. (D) U937 cells overexpressing wild type Bim or their empty vector control (pcDNA3.1) were treated with sorafenib (7.5 μM) and obatoclax (1.5 μM) for 24 h after which the percentage of dead cells was determined using the 7-AAD assay. * = significantly greater than values obtained for pcDNA3.1 cells (p < 0.05). (E) U937 cells in which Noxa was stably knocked down with shRNA and their control counterpart shGFP-transfected cells were exposed to sorafenib and obatoclax for 48 h after which the extent of cell death was determined using the 7-AAD assay. * = not significantly different from values obtained for shGFP-transfected cells (P > 0.05). Alternatively, cells were lysed and protein lysates subjected to Western blot analysis to monitor down-regulation of Noxa, and caspase-3 activation by Western blot analysis (F).

Figure 6. Role of autophagy in sorafenib/obatoclax-mediated lethality. (A) U937 cells were exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) for the designated intervals after which protein lysates were prepared and subjected to Western blot analysis. (B) U937 cells were stably transfected with LC3-EGFP, and EGFP positive cells were sorted by FACS and exposed to
sorafenib and obatoclax for 6 h. Cells were then fixed and subjected to confocal microscopy as described in Methods. (C) Representative images with confocal microscopy of co-localized LC3-GFP (Green) and LAMP1 (red) in U937 cells following 6-h treatment with sorafenib (7.5 μM) and obatoclax (1.5 μM). Enlarged images of outlined areas are shown in Supplemental Figure 5B. (D) U937 cells were pretreated with 3-MA (2.5 mM), chloroquine (CQ; 40 μM), or bafilomycin A (BAF; 75 nM) for 30 min, and exposed to sorafenib (7.5 μM) and obatoclax (1.5 μM) alone or in combination for an additional 16 h after which the extent of cell death was monitored by the 7-AAD assay. * = significantly greater than values for cells not exposed to 3MA, CQ, or BAF; P < 0.01. Alternatively, protein lysates were prepared from the indicated samples and subjected to Western blot analysis (E). (F) U937 cells in which VPS34 was stably knocked down with lentiviruses-mediated shRNA and their control scrambled sequence counterparts (NC) were exposed to sorafenib (7.5 μM) ± obatoclax (1.5 μM) for 24 h, after which the extent of cell death was determined using the 7-AAD assay. * = significantly different from values obtained for control cells (P < 0.05).

Figure 7. In vivo anti-leukemic activity of combined treatment with sorafenib and obatoclax. Nude mice were subcutaneously injected with U937 cells and subjected to treatment with sorafenib (80 mg/kg) and obatoclax (3.5 mg/kg) alone or together as described in Methods. Tumor volumes were measured at the indicated intervals (A), and pictures of 2 representative tumors for each group were obtained after 8 days of treatment (B). Xenograft-bearing mice were treated with sorafenib and/or obatoclax by intramuscular administration twice over a 24-h interval, after which tumors were excised, and either subjected to TUNEL analysis assays (C), or lysed, and subjected to Western blot analysis (D). (E) Kaplan-Meyer survival plot for mice
treated with sorafenib and obatoclax alone or in combination. The data shown is representative of 3 separate experiments each involving 5 mice/condition. The survival curves differed significantly between sorafenib/obatoclax and various other treatments (P = 0.001 to 0.04; logrank test).
Fig. 3

A

<table>
<thead>
<tr>
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<tr>
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<tr>
<td>Sor/Ob</td>
<td>IgG</td>
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IP: Bax (6A7)
WB: Bax

B

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IP: Bak (Ab1)
WB: Bak

C

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</table>

IP: Bak (Ab1)
WB: Bak

C-Casp3
PARP

D

% dead cells

shGFP  shBax  shBak

* *
**Fig. 4**

### Panel A

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**Markers:**
- Mcl-1
- Bcl-xL
- Bcl-2
- Bim
- Noxa
- Tub

### Panel B

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**Markers:**
- Mcl-1
- Tub
- ERK1/2

### Panel C

**Graph:**
- % dead cells
- pCEP4
- Mcl-1

### Panel D

**Markers:**
- c-Casp-3
- PARP
- Tub

**Graph:**
- Mcl-1
- IgG

### Panel E

**Markers:**
- IP: Bim
- IB: Bcl-2
- IB: Bcl-xL

**Graph:**
- Time (h) 0 8 24 8 24 8 24
Fig. 5

A  shGFP  shBim#1  shBim#2
Sor/Ob - + - + - +
Bim
C-casp3
Cyto-c
PARP
Tub

B  shGFP  shBim#1  shBim#2
Sor/Ob - + - + - +

C  % dead cells

D  pcDNA3.1  Bim
Bim
Tub
% dead cells

E  shGFP  shNoxa
shGFP  shNoxa

F  shGFP  shNoxa
shGFP  shNoxa

% dead cells

C  Sor  Ob  Sor  Ob
Sor/Ob - + - + - +

*  *  *
Fig. 6

A

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B

C

Lc3-GFP  LAMP1  Merge

D

% dead cells

E

F

VPS34  Tub
Fig. 7

A. Tumor volume (mm$^3$) over time (days).

B. Control, Sor, Ob, Sor/Ob.

C. DAPI, TUNEL, Merge.

D. Mcl-1, C-Casp-3, C-PARP, ERK1/2.

E. % survival over treatment intervals (days).
Inhibition of Bcl-2 anti-apoptotic members by obatoclax potently enhances sorafenib-induced apoptosis in human myeloid leukemia cells through a Bim-dependent process.

Mohamed Rahmani, Mandy Mayo Aust, Elisa Attkisson, David C. Williams, Jr., Andrea Ferreira-Gonzalez and Steven Grant