Obatoclax Interacts Synergistically with the Irreversible Proteasome Inhibitor Carfilzomib in GC- and ABC-DLBCL Cells In Vitro and In Vivo

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Abstract

Interactions between the irreversible proteasome inhibitor carfilzomib and the pan-BH3 mimetic obatoclax were examined in germinal center (GC)- and activated B-cell–diffuse large B-cell lymphoma (ABC-DLBCL) cells. Cotreatment with minimally toxic concentrations of carfilzomib (i.e., 2–6 nmol/L) and subtoxic concentrations of obatoclax (0.05–2.0 μmol/L) synergistically increased apoptosis in multiple DLBCL cell lines and increased lethality toward primary human DLBCL but not normal CD34+ cells. Synergistic interactions were associated with sharp increases in caspase-3 activation, PARP cleavage, p-JNK induction, upregulation of Noxa, and AKT dephosphorylation. Combined treatment also diminished carfilzomib–mediated Mcl-1 upregulation whereas immunoprecipitation analysis revealed reduced associations between Bak and Mcl-1/Bcl-xL and Bim and Mcl-1. The carfilzomib/obatoclax regimen triggered translocation, conformational change, and dimerization of Bax and activation of Bak. Genetic interruption of c-jun-NH2-kinase (JNK) and Noxa by short hairpin RNA knockdown, ectopic Mcl-1 expression, or enforced activation of AKT significantly attenuated carfilzomib/obatoclax–mediated apoptosis. Notably, coadministration of carfilzomib/obatoclax sharply increased apoptosis in multiple bortezomib-resistant DLBCL models. Finally, in vivo administration of carfilzomib and obatoclax to mice inoculated with SUDHL4 cells substantially suppressed tumor growth, sharply increased apoptosis in multiple bortezomib-resistant DLBCL models. Together, these findings argue that a strategy combining carfilzomib and obatoclax warrants attention in DLBCL. Mol Cancer Ther; 11(5); 1122–32. ©2012 AACR.

Introduction

Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 30% of all non–Hodgkin lymphoma in adults (1). Treatment options for patients have improved and median survival of patients with advanced stage DLBCL now exceeds 8 years; however, at least 25% to 30% of patients experience recurrence (2). Consequently, newer and more effective therapeutic strategies are clearly needed. Genetic profiling of DLBCL has led to the characterization of distinct DLBCL subtypes, that is, germinal center (GC-DLBCL), activated B-cell (ABC-DLBCL), and primary mediastinal (PM-DLBCL), which differ significantly with respect to their reliance on specific signaling pathways, biologic characteristics, and responses to current standard therapies (3).

The proteasome inhibitor bortezomib (Velcade), which binds reversibly to the 20S proteasome, has been approved for the treatment of refractory multiple myeloma and mantle cell lymphoma (MCL; refs. 4, 5). In contrast, single-agent activity of bortezomib in DLBCL is limited (6). However, addition of bortezomib to the DA-EPOCH regimen improved clinical outcomes in patients with ABC-DLBCL but not in other subtypes (7). Consequently, development of strategies active against both GC and ABC subtypes remains the subject of major interest. Carfilzomib, an irreversible proteasome inhibitor, exerts preclinical activity against bortezomib-resistant cells (8), and preliminary results suggest efficacy in heavily pretreated patients with multiple myeloma, including some who have previously received bortezomib (9). The mechanisms by which proteasome inhibitors kill transformed cells are unknown, but have been attributed to generation of reactive oxygen species (ROS; refs. 10, 11), endoplasmic reticulum (ER) stress (11), activation of the stress-related JNK (c-jun-NH2-kinase) pathway (12), and inhibition of NFκB–dependent pathways, among others (13, 14).
Dysregulation Bcl-2 family members occurs in numerous cancers, including lymphoma (15), prompting the development of small-molecule inhibitors, which bind to the BH3 hydrophobic–binding pocket of the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1. This has led to the development of BH3-mimetics such as ABT-737, which binds to Bcl-2, Bcl-xL, and A1, but not Mcl-1 (16), and GX15-070 (obatoclax), a pan-Bcl-2 inhibitor, which also binds to and inactivates Mcl-1 (17). These agents are currently under evaluation in diverse hematopoietic malignancies, including lymphoma (18).

Findings from several laboratories, including our own, have showed synergistic interactions between proteasome inhibitors (e.g., bortezomib) and BH3-mimetics (e.g., HA14-I) in malignant hematopoietic cells, including multiple myeloma, MCL, and DLBCL (19–21). Furthermore, enhanced lethality has been observed when ABT-737 was combined with bortezomib or carfilzomib in various lymphoma cell types (22, 23). However, the mechanisms by which such interactions occur have not yet been elucidated in DLBCL cells. Furthermore, information concerning the effects of irreversible proteasome inhibitors such as carfilzomib on the response of bortezomib-resistant cells to BH3-mimetics targeting Mcl-1 is lacking. Such information is relevant in light of evidence implicating Mcl-1 in proteasome inhibitor resistance (24).

The present goal was to determine whether and by what mechanisms carfilzomib and obatoclax might lead to increased cell death in GC- and ABC-DLBCL cells, including those resistant to bortezomib, and to define the mechanisms by which interactions occurred. Our results indicate that these agents interact synergistically in both GC- and ABC-DLBCL subtypes and in bortezomib-resistant cells. Furthermore, potentiation of carfilzomib lethality by obatoclax depends functionally on activation of the stress kinase JNK, induction of Noxa, downregulation of AKT phosphorylation and release of proapoptotic protein Bim from sequestration by Mcl-1, and release of Bak from both Mcl-1 and Bcl-xL. Significantly, the carfilzomib/obatoclax regimen displays pronounced activity in an in vivo DLBCL xenograft model. Together, these findings provide a mechanistic framework for combining carfilzomib with obatoclax in DLBCL.

Materials and Methods

Cells

SUDHL16, SUDHL4 (both GC), OCI-LY10, OCI-LY3 (both ABC), and primary DLBCL cells were obtained and authenticated as previously described (13). Bortezomib-resistant SUDHL16-10BR (GC), OCI-LY10-40BR (ABC) were generated as described in Supplementary Methods (13). SUDHL16-shJNK cells were generated by electroporation (Amaxa) with buffer L as described previously (13). Cells ectopically expressing activated AKT were generated by transfecting pUSE-myr-AKT1 cDNA (Upstate) into SUDHL16 cells as before (13). Stable clones were selected by serial dilution using antibiotics (13). Five drug-resistant clones were selected for each type (sh-JNK and AKT-CA). They validated functionally significance and results using 2 or 3 randomly selected clones are shown. All experiments were conducted with logarithmically growing cells (e.g., 4.0–5.0 × 10^6 cells/mL) within passages 6 to 24 to ensure uniform responses. Mycoplasma tests were uniformly negative (MycoAlert Mycoplasma Detection Kit, Lonza, Inc.). The construct pcDNA3.1-Mcl-1 was a generous gift from Dr. R.W. Craig (Dartmouth Medical School, Hanover, NH) and used to express Mcl-1 in SUDHL4 cells by transient transfection. HuSH29 mer short hairpin RNA (shRNA) constructs against NOXA1 in a pRFP-C-RS vector from Origene Technologies, (catalog no TF31134) were used to knockdown NOXA in SUDHL4 cells through transient transfection. Cell lines were authenticated by short tandem repeat (STR) DNA fingerprinting using the AmpFISTR Identifiler kit (Applied Biosystems). The STR profiles were compared with known American Type Culture Collection (ATCC) database and to the German Collection of Microorganisms and Cell Cultures database (http://www.dsmz.de/).

Transient transfection

Transient transfection of SUDHL4 cells used an Amaxa Nucleofector shuttle apparatus (Cologne) as per protocol in 96-well plate mode (details in Supplementary Methods).

Reagents

Carfilzomib was provided by Onyx Pharmaceuticals. Bortezomib (Velcade) was from Millennium Pharmaceuticals. Obatoclax (formerly GX15-070) was from Cephalon. 7-Aminominoenaminycin D (7-AAD) was purchased from Sigma-Aldrich. All agents were formulated in dimethyl sulfoxide. Structures of carfilzomib and obatoclax were illustrated in Supplementary Fig. S1.

Experimental format

Cells were cultured as described earlier (13), treated with drugs and prepared for analysis as described later.

Assessment of cell death and apoptosis

Cell viability was monitored by flow cytometry with 7-AAD staining as before (13) and in some cases validated by Trypan blue staining.

Collection of CD34^+ cells

These studies have been approved by the Investigational Review Board of Virginia Commonwealth University (Richmond, VA; IRB #HM12433 for patient samples and #03340 for normals). CD34^+ cells were isolated using an immunomagnetic bead separation technique as described in Supplementary Methods (13).

Western blot analysis

Western blot samples were prepared from whole-cell pellets as described (13). Sources of primary antibodies
were as follows: p-AKT, AKT1, p-JNK, JNK1, p-p44/42, p-p44/42, BID, Bcl-xL were from Santa Cruz Biotechnology; cleaved caspase-3, P-histone-H3.X were from Cell Signaling Technology; PARP (C-2–10) was from Upstate Biotechnology; and tubulin was from Oncogene. Actin antibodies were purchased from Sigma. Bcl-2 antibodies were from Dako. McI-1 antibodies were purchased from BD BioScience.

**Immunoprecipitation**
Analyses of protein complexes by immunoprecipitation including conformationally changed Bak were conducted with CHAPS lysis buffer and immunomagnetic Dynabeads M-450 microspheres (Invitrogen; ref. 25).

**Bax/BAK conformational change**
Drug-treated cells were subjected to cellular fractionation as described (26) and are described in detail in Supplementary Methods.

**Bax oligomerization**
Cells were treated with various agents and Bax oligomerization was studied as described in Supplementary Methods.

**Animal studies**
Animal studies were conducted using Beige-nude-XID mice (NIH-III; Charles River). A total of 10 × 10⁶ SUDHL4 cells were pelleted, washed twice with 1× PBS, and injected subcutaneously into the right flank. Once the tumors were visible, mice were grouped into 4 separate sets with 5 to 6 mice in each set. The control group was treated with vehicle alone (10 mmol/L citrate buffer and 5% dextrose solution) and the remaining 3 groups were treated with carfilzomib ± obatoclax. Carfilzomib was administered via tail vein (i.v. twice weekly) injection of each single agent were tested for synergy using the Laska model-free (27, 28) test for synergy, implemented with unequal variance 0.05 level 2-sample t tests in R, and characterized via contour plots of the dose–response surface. Synergism was also evaluated by median dose effect analysis with a software program (CalcuSyn, Biosoft; ref. 29). Cells were treated with carfilzomib and obatoclax at fixed concentration ratios (1:375) for 48 hours, and cell death was monitored by 7-AAD staining and flow cytometry. Combination index (CI) values and isobolograms were computed with CalcuSyn software. CI values less than 1.0 denote synergism. Survival functions were estimated using Kaplan–Meier curves and compared using the log-rank test.

**Results**

**Carfilzomib and obatoclax interact synergistically to induce apoptosis in GC- and ABC-DLBCL cells**
Whereas exposure (24 hours) to 150 to 250 nmol/L obatoclax alone minimally induced apoptosis in GC-DLBCL SUDHL16 cells, coexposure to carfilzomib concentrations as low as 1.5 nmol/L significantly increased apoptosis, and concentrations ≥3.5 nmol/L resulted in apoptosis in the large majority of cells (Fig. 1A). Conversely, 2 to 3 nmol/L carfilzomib by itself was minimally toxic, but coadministration of obatoclax concentrations as low as 100 to 200 nmol/L significantly increased cell death, and concentrations ≥250 nmol/L resulted in a pronounced increase in apoptosis (Fig. 1B). Time course studies revealed a sharp increase in apoptosis in cells exposed to carfilzomib and obatoclax starting at 24 hours, increasing further over the ensuing 48 hours (Fig. 1C). Two approaches (the Laska model-free test and median dose effect analysis) were used to evaluate synergism. For the Laska test of 56 data sets, several dose combinations of carfilzomib and obatoclax with nonzero concentrations of both drugs were evaluated. The 8 combinations lying below the straight line connecting the highest concentration of each single agent were tested for synergy using the Laska method, conservatively extended for the most extreme testable combination that did not fall directly on any straight line connecting single-agent doses by comparison. Synergy occurred at all 8 testable combinations, with P values ranging from less than 0.0001 to 0.0027 (Fig. 1D). Median dose effect analysis revealed CI values less than 1.0, indicating synergistic interactions (Fig. 1E). On the basis of this analysis, mean carfilzomib (2.5 nmol/L) and obatoclax (200 nmol/L) concentrations were used for subsequent mechanistic studies. Similar synergistic interactions were observed in SUDHL4 (Supplementary Fig. S2) and ABC-DLBCL cells (data not shown). A pronounced increase in apoptosis also occurred when carfilzomib was combined with another BH3-mimetic, HA14-1 (19) in OCI-LY10 (ABC subtype) and OCI-LY3 (ABC subtype) cells (Fig. 1F). Finally, significant increases in lethality were observed when primary DLBCL cells (one GC- and one ABC subtype) cells were coexposed to carfilzomib and obatoclax (Fig. 1G). However, coexposure to carfilzomib and obatoclax did not significantly potentiate lethality compared with single-agent treatment in normal CD34+ bone marrow cells (Fig. 1H).
Carfilzomib and Obatoclax in DLBCL Cells

Figure 1. Carfilzomib/obatoclax cotreatment synergistically induces cell death in DLBCL lymphoma lines and primary lymphoma cells but not in normal cells. A, SUDHL16 cells were treated (48 hours) with carfilzomib (CFZ; 2.0–4.0 nmol/L) ± obatoclax (Obato; 150–250 nmol/L), after which apoptosis was monitored by 7-AAD staining and normalized to controls. For all studies: values, means for 3 experiments carried out in triplicate.

B, SUDHL16 cells were treated (48 hours) with 100 to 300 nmol/L obatoclax ± carfilzomib (2.0–3.0 nmol/L), followed by 7-AAD staining. C, time course of SUDHL16 cell death (7–AAD) following obatoclax/carfilzomib exposure. D, synergistic interactions between carfilzomib and obatoclax at various concentrations in SHDHL16 cells were tested using the Laska model-free test for synergy as in Materials and Methods. Synergism was observed for all 8 combinations tested with $P$ values ranging from less than 0.001 to 0.0027. E, CI values were determined with CalcuSyn software using fixed drug ratios of carfilzomib and obatoclax (1:80) in SUDHL16 cells (f) OCI-LY10 and OCI-LY3 cells were treated (48 hours) with carfilzomib (5.0 and 4.0 nmol/L, respectively) ± obatoclax (50 and 75 nmol/L, respectively); cell death was monitored by 7-AAD/DiOC6 staining. G, two primary human DLBCL (one ABC and other GC subtype) specimens were isolated and treated with carfilzomib and obatoclax (1:80) in SUDHL16 cells (F) OCI-LY10 and OCI-LY3 cells were treated (48 hours) with carfilzomib (2 and 100 nmol/L for ABC and GC subtypes, respectively) ± obatoclax (500 nmol/L). Cell death was monitored by 7-AAD staining and normalized to controls. Viability of untreated primary specimens was 80% (H) CD34+ cells were exposed (48 hours) to carfilzomib (10–20 nmol/L) ± obatoclax (500 nmol/L). Cell death was monitored by 7-AAD staining and normalized to controls. For all studies: values, means for 3 experiments carried out in triplicate ± SD. For A–C, F, G, values significantly greater than those for carfilzomib or obatoclax treatment alone; $P < 0.01$. Cont, control.

Carfilzomib/obatoclax exposure activates JNK, inactivates AKT, upregulates Noxa, and induces γH2A.X in DLBCL cells

Exposure of SUDHL16 cells to carfilzomib (24 hours, 2.5 nmol/L) or obatoclax (200 nmol/L) alone minimally induced caspase-3 cleavage and PARP degradation (Fig. 2A). In contrast, coadministration sharply increased caspase-3 activation and PARP degradation, accompanied by a marked increase in phosphorylation of the stress-related JNK kinase and c-Jun (Fig. 2B). Similar effects were observed in SHDHL4 cells (data not shown). Combined treatment also reduced expression of p-AKT but not total AKT expression (Fig. 2C). In contrast, obatoclax increased p-ERK1/2, an effect that was attenuated by carfilzomib coexposure.

Individual or combined exposure had little effect on expression of Bid, Bcl-xL, or Bcl-2, a Bcl-2 cleavage fragment was noted with the combination (Fig. 2D). Consistent with results involving bortezzomib (24, 30), carfilzomib modestly but discernibly increased Mcl-1 levels (Fig. 2E). Time course analysis showed an increase in Mcl-1 levels appreciable after 6-hour exposure to 2.5 nmol/L carfilzomib and pronounced at intervals ≥12 hours (Fig. 2E). Notably, obatoclax sharply decreased Mcl-1 expression and attenuated carfilzomib-mediated downregulation. These events were accompanied by a pronounced increase in expression of γH2A.X, reflecting double-stranded DNA breaks (31, 32). Similar results were observed in OCI-LY10 cells (Fig. 2F). Finally, carfilzomib (2–4 nmol/L, 24 hours) diminished NF-κB activity in both SUDHL16 and OCY-LY10 cells by approximately 30% to 40% but this effect was not enhanced by obatoclax (Supplementary Fig. S5).

In view of evidence that obatoclax triggers autophagy in malignant hematopoietic cells (33), the effects on autophagy were examined in SUDHL16 cells. Obatoclax (200 nmol/L) induced autophagy in these cells, manifested by processing of LC3-I to LC3-II accompanied by...
degradation of p62 (data not shown). However, no changes in autophagy were observed with carfilzomib, arguing against the possibility that perturbations in autophagy played a major role in lethality.

**Combined exposure of DLBCL cells to carfilzomib and obatoclax diminishes the association of Mcl-1 to Bak and Bim and triggers Bak and Bax activation**

While exposure (24 hours) of SUDHL16 cells to carfilzomib (2.5 nmol/L) or 200 nmol/L obatoclax individually triggered Bak mitochondrial translocation, combined treatment resulted in a very pronounced increase (Supplementary Fig. S4A). Consistent with previous reports, Bak was localized to the mitochondria (34) and levels increased modestly following obatoclax exposure (Supplementary Fig. S4A). Carfilzomib and to a lesser extent obatoclax triggered Bak conformational change/activation, whereas combined treatment induced a marked increase (Supplementary Fig. S4B). In contrast, obatoclax but not carfilzomib modestly induced Bak conformational change, whereas effects with combined treatment were very pronounced. Finally, Bax dimerization sharply increased following combined carfilzomib/obatoclax exposure (Supplementary Fig. S4C).

Immunoprecipitation studies revealed that obatoclax but not carfilzomib diminished Mcl-1/Bim binding, whereas combined treatment dramatically reduced this association (Supplementary Fig. S4D). Moreover, obatoclax strongly diminished the Mcl-1/Bak association (Supplementary Fig. S4D). Individual exposure to carfilzomib or obatoclax had little effect on Bcl-xL/Bak binding, whereas combined treatment substantially blocked this association. Finally, reverse immunoprecipitation analysis confirmed the pronounced ability of the carfilzomib/obatoclax regimen to antagonize Mcl-1 binding to Bak and Bim and Bcl-xL to Bak (Supplementary Fig. S4E).

**JNK activation and AKT inactivation play significant functional roles in obatoclax/carfilzomib lethality in DLBCL cells**

To assess the functional significance of JNK activation and AKT inactivation in obatoclax/carfilzomib lethality, SUDHL16 cells were stably transfected with scrambled sequence or JNK shRNA constructs (CL8

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**Figure 2.** Carfilzomib/obatoclax coexposure activates JNK, inactivates AKT, upregulates Noxa, and induces γH2A.X in DLBCL cells. SUDHL16 cells were treated (24 hours) with carfilzomib (2.5 nmol/L) or obatoclax (200 nmol/L). A–D, expression of the indicated proteins was determined by Western blotting. E, SUDHL16 cells were treated with 2.5 nmol/L carfilzomib and Mcl-1 determined by Western blotting. Each lane was loaded with 20 µg of protein; blots were stripped and reprobed with antibodies directed against tubulin to ensure equivalent loading and transfer. Results are representative of 3 separate experiments (F). OCI-Ly10 cells were treated with carfilzomib (4.0 nmol/L) or obatoclax (50 nmol/L) for 24 hours and expression of the indicated proteins determined by Western blotting. BID, twice daily; Cont, control; CF, cleaved form.
or CL12). Antisense clones displayed a partial but clearly discernible reduction in JNK expression compared with scrambled sequence controls (Fig. 3A). Furthermore, following obatoclax/carfilzomib exposure, shRNA clones exhibited a partial but significant reduction in apoptosis ($P < 0.05$). Western blot analysis documented diminished p-JNK induction and caspase-3 cleavage in CL8 clones compared with controls (Fig. 3B).

Parallel studies were conducted using SUDHL16 cells ectopically expressing constitutively active AKT. Two clones (AKT cl3 and cl5) displayed significantly increased phosphorylation of the AKT target GSKα/β compared with empty vector controls (Fig. 3C, top) and exhibited partial but significant ($P < 0.05$) reductions in carfilzomib/obatoclax lethality compared with controls (Fig. 3C, bottom). They also displayed diminished inhibition of AKT phosphorylation, PARP cleavage, and caspase-3 activation compared with controls following carfilzomib/obatoclax exposure (Fig. 3D), arguing for a functional role for JNK activation and AKT inactivation in carfilzomib/obatoclax lethality in DLBCL cells.

**Figure 3.** Genetic interruption of JNK and AKT significantly diminishes carfilzomib/obatoclax lethality. A, SUDHL16-JNK shRNA or scrambled (scram) sequence vectors or empty vector (pUSE) were transfected into SUDHL16 and exposed (48 hours) to carfilzomib (2.5 nmol/L) + obatoclax (150 nmol/L), after which, cell death was monitored by 7-AAD. Inset, expression of JNK protein in SUDHL16-scrambled sequence and shJNK clones. B, following 24-hour treatment as in A, protein was monitored by Western blotting (C). SUDHL16 cells were stably transfected with constitutively active (myristolated) AKT constructs (AKT cl.3 and 5) or empty vector (pUSE) and exposed (48 hours) to carfilzomib (3.0 nmol/L) + obatoclax (150 nmol/L), after which, cell death was monitored by 7-AAD. Inset, Western blot analysis showing expression of AKT and p-GSK-3α/β in empty vector control and AKT clones. D, cells were treated as described earlier in C for 24 hours, after which Western blot analysis was conducted to monitor expression of the indicated proteins. For A and C: * significantly less than values obtained for carfilzomib + obatoclax treatment in SUDHL16-scrambled sequence or SUDHL16-expressing pUSE cells; $P < 0.05$. Cont, control.

Noxa upregulation and Mcl-1 downregulation play functional roles in carfilzomib/obatoclax lethality in DLBCL cells

SUDHL4 cells transiently expressing Noxa shRNA displayed a clear reduction in Noxa expression following exposure (24 hours) to either bortezomib (data not shown) or carfilzomib/obatoclax (Fig. 4A, top), associated with a modest but significant reduction in carfilzomib/obatoclax-mediated apoptosis ($P < 0.05$ compared with control pRFP-C-RS-scrambled sequence cells; Fig. 4A, bottom). Carfilzomib/obatoclax exposure was associated with significantly reduced PARP and caspase-3 cleavage in Noxa shRNA cells compared with their control counterparts (Fig. 4B).

Parallel studies were conducted with SUDHL4 cells ectopically expressing Mcl-1 by transient transfection. As shown in Fig. 4C (top), Mcl-1 was overexpressed following transfection of pcDNA3.1-Mcl-1 cDNA versus empty vector control pcDNA3.1 (Fig. 4C, top), associated with a significant reduction in apoptosis following carfilzomib/obatoclax exposure ($P < 0.05$; Fig. 4C, bottom). Notably, carfilzomib/obatoclax-treated Mcl-1-overexpressing cells displayed a marked increase in Mcl-1

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Noxa upregulation and Mcl-1 downregulation play functional roles in potentiating carfilzomib/obatoclax lethality in DLBCL cells. A, SUDHL4 cells were transiently transfected with a Noxa construct or an empty vector (pRFP-C-RS) and exposed (48 hours) to carfilzomib (1.5 nmol/L) + obatoclax (1.0 μmol/L), after which cell death was monitored by 7-AAD. Inset, Western blot analysis showing expression of Noxa in empty vector control and Noxa-transfected cells following carfilzomib (1.5 nmol/L) + obatoclax (1.0 μmol/L) treatment. B, cells were treated as in A for 24 hours, after which expression of the indicated proteins was monitored. C, SUDHL4 cells were transiently transfected with a Mcl-1 construct or an empty vector (pcDNA3.1) and exposed (48 hours) to carfilzomib (2.0 nmol/L) + obatoclax (1.0 μmol/L) for 48 hours. Cell death was monitored by 7-AAD. Inset, Western blot analysis showing expression of Mcl-1 in empty vector control and transiently transfected cells. D, cells were treated as in C for 24 hours, after which Western blot analysis was conducted to monitor protein expression. Immunoprecipitation was used to characterize the association between Bim and Mcl-1. For A and C: *, significantly less than values obtained for carfilzomib + obatoclax treatment in SUDHL16-scrambled sequence or SUDHL16-Mcl-1. For A and C: †, significantly less than values obtained for carfilzomib + obatoclax treatment in SUDHL16-Mcl-1 controls; P < 0.05. Cont, control.

**Figure 4.** Noxa upregulation and Mcl-1 downregulation play functional roles in potentiating carfilzomib/obatoclax lethality in DLBCL cells. A, SUDHL4 cells were transiently transfected with a Noxa construct or an empty vector (pRFP-C-RS) and exposed (48 hours) to carfilzomib (1.5 nmol/L) + obatoclax (1.0 μmol/L), after which cell death was monitored by 7-AAD. Inset, Western blot analysis showing expression of Noxa in empty vector control and Noxa-transfected cells following carfilzomib (1.5 nmol/L) + obatoclax (1.0 μmol/L) treatment. B, cells were treated as in A for 24 hours, after which expression of the indicated proteins was monitored. C, SUDHL4 cells were transiently transfected with a Mcl-1 construct or an empty vector (pcDNA3.1) and exposed (48 hours) to carfilzomib (2.0 nmol/L) + obatoclax (1.0 μmol/L) for 48 hours. Cell death was monitored by 7-AAD. Inset, Western blot analysis showing expression of Mcl-1 in empty vector control and transiently transfected cells. D, cells were treated as in C for 24 hours, after which Western blot analysis was conducted to monitor protein expression. Immunoprecipitation was used to characterize the association between Bim and Mcl-1. For A and C: *, significantly less than values obtained for carfilzomib + obatoclax treatment in SUDHL16-scrambled sequence or SUDHL16-Mcl-1 controls; P < 0.05. Cont, control.

**Carfilzomib and obatoclax interact synergistically in bortezomib-resistant GC- and ABC-DLBCL cells**

To assess the efficacy of the carfilzomib/obatoclax regimen in proteasome inhibitor–resistant cells, bortezomib-resistant SUDHL16-10BR and OCI-LY-40BR cells were used (13, 20). These cells exhibit no lethality in the presence of 10 or 40 nmol/L bortezomib, respectively, whereas essentially 100% of parental cells die under these conditions. Coadministration of carfilzomib (concentrations, 5–20 nmol/L) and obatoclax (concentrations, 100–250 nmol/L), which were minimally toxic by themselves, sharply increased lethality when coadministered (Fig. 5A). CI values were also substantially below 1.0, indicating a synergistic interactions (Fig. 5B). Combined treatment markedly increased caspase-3 and PARP cleavage in SUDHL16-10BR cells, accompanied by Noxa upregulation and increased γH2A.X expression (Fig. 5C), as in the case of sensitive parental cells. Similar results were obtained with OCI-LY-40BR cells (data not shown). Finally, exposure of SUDHL16-10BR and OCI-LY10-40BR cells to carfilzomib/obatoclax reduced Mcl-1 coimmunoprecipitating with Bim and Bak, and diminished coimmunoprecipitation of Bcl-xL with Bak compared with single-agent treatment, as in parental cells (Fig. 5D).

**The carfilzomib/obatoclax regimen displays in vivo activity in a DLBCL xenograft model**

To evaluate the *in vivo* implications of these findings, a previously described SUDHL4 xenograft model was used (13). Whereas obatoclax (3.0 mg/kg) had little effect on tumor growth, carfilzomib (2.0 mg/kg) by itself significantly reduced tumor size (Fig. 6A). However, combined treatment resulted in minimal tumor growth, an effect significantly greater than that observed with either agent alone (P < 0.05). IVIS imaging of luciferase-expressing tumor cells confirmed the marked reduction in tumor growth with combined therapy (Fig. 6B). Kaplan–Meier
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Figure 5. The carfilzomib/obatoclax regimen potently induces apoptosis in bortezomib-resistant SUDHL16-10BR and OCI-LY10-40BR cells. A, SUDHL16-10BR and OCI-LY10-40BR cells were treated (48 hours) with minimally toxic concentrations of carfilzomib and obatoclax. Concentrations were as follows: SUDHL16-10BR, carfilzomib (5 nmol/L) ± obatoclax (250 nmol/L); OCI-LY10-40BR, carfilzomib (20 nmol/L) ± obatoclax (100 nmol/L); Cell death was monitored by 7-AAD. B, median dose effect analysis yielded CI values less than 1.0 denoting synergistic interactions. C, SUDHL16-10BR cells were exposed (24 hours) to carfilzomib and obatoclax as in A, after which, Western blot analysis was conducted. D, SUDHL16-10BR and OCI-LY10-40BR cells were treated with carfilzomib and obatoclax for 24 hours as in A after which, associations between Mcl-1 and Bak and BIM, Bcl-xL with Bak were characterized by immunoprecipitation followed by Western blotting. Cont, control.

Discussion

Because proteasome inhibitors exert pleiotropic effects (35), they represent attractive candidates for combination with other targeted agents. BH3-mimetics recapitulate the actions of BH3-only proteins and circumvent the actions of antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, and in some cases Mcl-1 (16–18, 23, 36), promoting activation of Bak and Bax, accompanied by Bax mitochondrial translocation, culminating in mitochondrial injury and apoptosis. Several groups, including our own, initially reported that BH3-mimetics interacted synergistically with bortezomib in human multiple myeloma cells (19), and more recently, such findings have been extended to non-Hodgkin lymphoma models (20). The present studies were prompted by several considerations. First, single-agent activity of bortezomib in DLBCL is limited (6) and showed activity in only ABC-DLBCL when combined with chemotherapy (7). It is possible that the irreversible proteasome inhibitor carfilzomib, which is active in bortezomib-resistant models (8, 13, 25), might represent an effective alternative in combination strategies, particularly in bortezomib-resistant cells. Moreover, the BH3-mimetic obatoclax, in contrast to certain other BH3-mimetics, for example, ABT-737 (16), downregulates and inactivates Mcl-1 (17, 36), a protein implicated in proteasome inhibitor resistance (24). Finally, mechanisms underlying carfilzomib/obatoclax interactions have not been defined in vitro or in vivo. The present findings show that a dual approach involving the second-generation proteasome inhibitor carfilzomib and the pan-BH3 inhibitor obatoclax is effective against GC- and ABC-DLBCL cells, including bortezomib-resistant cells and displays significant in vivo activity in a xenograft model. They also argue that perturbations in AKT and JNK, as well as Bcl-2 family proteins (e.g., Noxa upregulation, dissociation of Bcl-2/Bcl-xL/Mcl-1 from proapoptotic effectors) contribute to synergistic interactions.

While proteasome inhibitors trigger accumulation of prosapoptotic proteins (e.g., Bim), they may also...
upregulate antiapoptotic proteins, particularly Mcl-1 (35), implicated in bortezomib resistance of multiple myeloma (30). Obatoclax, which both disrupts the function of Mcl-1 (37) and triggers upregulation of Noxa (38), a protein capable of downregulating Mcl-1 (30), potentiates bortezomib lethality in multiple myeloma, MCL, and other models (17, 19, 20). Here, carfilzomib alone upregulated Mcl-1, an event prevented by obatoclax coadministration. The finding that ectopic expression of Mcl-1 attenuated carfilzomib/obatoclax-mediated cell death argues that attenuating Mcl-1 accumulation contributes functionally to lethality. Consistent with findings in multiple myeloma (37) and MCL cells (38), obatoclax promoted Mcl-1/Bak dissociation in DLBCL cells. However, this phenomenon was more marked with obatoclax/carfilzomib coexposure and occurred at considerably lower obatoclax concentrations than previously reported, for example, approximately 200 mmol/L versus ≥1 μmol/L, possibly reflecting the pronounced Mcl-1 downregulation in carfilzomib/obatoclax-treated cells. Furthermore, cells exposed to obatoclax, particularly with carfilzomib, displayed a striking reduction in associations between Bcl-xL and Bak and between Mcl-1 and Bim. Disruption of the Bcl-xL/Bak association cooperates with release of Bak from Mcl-1 to trigger Bak activation and apoptosis (39). Moreover, Bim release from Mcl-1 by Noxa has been implicated in proapoptotic inhibitors lethality in myeloma cells (30), and Noxa induction contributes significantly to obatoclax/bortezomib interactions in MCL cells (38). In contrast, a functional role for Noxa in analogous interactions in DLBCL cells has not yet been defined. It is possible that each of these event, for example, upregulation of Noxa, untethering of Bak from both Mcl-1 and Bcl-xL, and release of Bim from Mcl-1 contributes to the enhanced lethality of the carfilzomib/obatoclax regimen in DLBCL cells. Finally, the observations that Mcl-1 overexpression or Noxa knockdown diminished carfilzomib/obatoclax-induced apoptosis argues that perturbations in these proteins contribute to the enhanced lethality.

The mitogen-activated protein (MAP) kinase JNK is activated by diverse stresses, and generally exerts proapoptotic effects, in contrast to extracellular signal-regulated kinase (ERK)1/2, which principally plays a cytoprotective role (40). JNK activation has been observed in multiple myeloma cells simultaneously exposed to bortezomib and BH3-mimetics (12, 41). Consistent with these findings, treatment of DLBCL cells with carfilzomib/obatoclax sharply increased JNK activation. In addition, combined treatment also markedly increased AH2A.X expression, reflecting double-stranded DNA breaks (31). In this context, proteasome inhibition interferes with DNA repair processes (31, 42), and MCL cells defective in DNA repair are particularly susceptible to obatoclax lethality (38). Together, these observations raise the possibility that the genotoxic effects of combined carfilzomib/obatoclax exposure triggers JNK activation and lethality. Finally, the cytoprotective activation of AKT in response to DNA damage has been described (43, 44). Whatever the mechanism of JNK activation and AKT inactivation, the findings that knockdown of JNK or enforced AKT activation diminish carfilzomib/obatoclax lethality argue that the former events contribute functionally to enhanced
lethality. A schematic diagram depicting potential mechanisms underlying synergistic interactions between carfilzomib and obatoclax is illustrated in supplementary Fig. S6.

Obatoclax induces an autophagy response in various malignant hematopoietic cells, including myeloma and leukemia (33, 45). In DLBCL cells, obatoclax induced autophagy by itself, but this phenomenon was not potentiated by carfilzomib. Consequently, perturbations in autophagy appear unlikely to play a major role in the enhanced lethality of the obatoclax/carfilzomib regimen.

Resistance to proteasome inhibitors such as bortezomib involves multiple mechanisms, including upregulation or mutation of proteasome subunits, induction of antioxidative defenses, or upregulation of antiapoptotic proteins such as Mcl-1, among others (46). Although carfilzomib exhibits significant activity against certain bortezomib-resistant cells (e.g., myeloma; refs. 8, 13), cross-resistance, that is, in DLBCL or MCL cells is incomplete (13, 25). Nevertheless, highly synergistic interactions were observed in multiple bortezomib-resistant-DLBCL lines following exposure to obatoclax and low (e.g., 5–18 nmol/L) concentrations of carfilzomib. Significantly, several mechanisms implicated in carfilzomib/obatoclax interactions in bortezomib-sensitive cells, that is, JNK activation, λH2A.X induction, release of Bak and Bim from Mcl-1, and Bak from Bcl-xL, were also observed in resistant cells exposed to these agents, albeit at slightly higher carfilzomib concentrations. Such findings suggest that increasing carfilzomib concentrations in bortezomib-resistant DLBCL cells may result in similar synergistic interactions with obatoclax as observed in sensitive counterparts and through analogous mechanisms.

In addition to in vitro interactions, carfilzomib/obatoclax coadministration in immunodeficient mice inoculated with DLBCL cells sharply reduced tumor cell growth accompanied by increased survival compared with single-agent treatment. Furthermore, several of the events implicated in in vitro synergism, for example, p-JNK upregulation and p-AKT downregulation occurred in excised tumors, suggesting that mechanisms underlying synergistic interactions in vitro may be operative in vivo. These findings also raise the possibility that one or more of the present observations (e.g., Noxa upregulation, JNK activation, AKT inactivation, λH2A.X induction) could serve as correlative response determinants in future clinical trials investigating this strategy. Finally, the observations that the obatoclax/carfilzomib regimen was active against multiple GC- and ABC-DLBCL lines, as well as bortezomib-resistant and primary cells, while exhibiting minimal increases in toxicity toward normal cells and intact animals, raise the possibility that this strategy might be of value in patients with refractory DLBCL. These considerations may be particularly relevant given the limited activity of bortezomib in DLBCL. Accordingly, plans for a phase I trial of carfilzomib and obatoclax in this patient population are in progress. As carfilzomib administered as a single agent has showed significant activity in patients with bortezomib refractory multiple myeloma (9), the present findings raise the possibility that the carfilzomib/obatoclax regimen may also warrant attention in this disease.

Disclosure of Potential Conflicts of Interest

R.I. Fisher is a consultant/ advisory board member for Roche, Millennium, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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 Writing, review, and/or revision of the manuscript: G. Dasmahapatra, D. Lembersky, D.R. Peterson, R.I. Fisher, J.W. Friedberg, P. Dest, S. Grant
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References


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Obatoclax Interacts Synergistically with the Irreversible Proteasome Inhibitor Carfilzomib in GC- and ABC-DLBCL Cells *In Vitro* and *In Vivo*

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