Obatoclax interacts synergistically with the irreversible proteasome inhibitor carfilzomib in GC- and ABC- DLBCL cells in vitro and in vivo

Girija Dasmahapatra, Dmitry Lembersky, Minkyeong P. Son, Hiral Patel, Derick Peterson, Elisa Attkisson, Richard I. Fisher, Jonathan W. Friedberg, Paul Dent, Steven Grant

Division of Hematology/Oncology, Department of Medicine, Virginia Commonwealth University, the James T Wilmot Cancer Center and the Departments of Medicine and Biostatistics, University of Rochester Medical Center, and the Massey Cancer Center and Virginia Institute of Molecular Medicine, Virginia Commonwealth University

Running Title: Carfilzomib and obatoclax in DLBCL cells

Key words: Carfilzomib, Obatoclax, DLBCL, NHL

Abbreviations list: Carfilzomib – CFZ, Obatoclax – Obato, Diffuse Large B-cell Lymphoma cells – DLBCL cells, GC – Germinal center, Activated B-cell-ABC

Financial support: SG, GD, and DL were supported by awards CA63753, CA93738, and CA100866 from the National Institutes of Health; award R6059-06 from the Leukemia and Lymphoma Society of America, the Multiple Myeloma Research Foundation, Myeloma Spore (P50CA142509) and the V Foundation. SG, GD, DL, MS, RF, and JF were supported by Lymphoma SPORE award 1P50 CA130805.
To whom reprint requests should be sent at the following address:

Steven Grant, M.D.
Division of Hematology/Oncology
Virginia Commonwealth University/Medical College of Virginia
MCV Station Box 230
Richmond, VA 23298
Phone: 804-828-5211
Fax: 804-828-8079
Email: stgrant@vcu.edu

Pages (text): 26
Abstract (words): 195
Text (words): 5805
Figures: 6
Supplemental Figures: 6
References: 46
Potential conflict of interest: No conflict of interest to report
ABSTRACT

Interactions between the irreversible proteasome inhibitor carfilzomib (CFZ) and the pan-BH3 mimetic obatoclax (Obato) were examined in GC- and ABC-DLBCL cells. Co-treatment with minimally toxic concentrations of CFZ (i.e., 2-6 nM) and sub-toxic concentrations of obato (0.05-2.0µM) 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, phospho-JNK induction, up-regulation of Noxa, and AKT dephosphorylation. Combined treatment also diminished CFZ-mediated Mcl-1 up-regulation while immunoprecipitation analysis revealed reduced associations between Bak and Mcl-1/Bcl-xL, and Bim and Mcl-1. The CFZ/Obato regimen triggered translocation, conformational change and dimerization of Bax and activation of Bak. Genetic interruption of JNK and Noxa by shRNA knockdown, ectopic Mcl-1 expression, or enforced activation of AKT significantly attenuated CFZ/Obato-mediated apoptosis. Notably, co-administration of CFZ/Obato sharply increased apoptosis in multiple bortezomib-resistant DLBCL models. Finally, in vivo administration of CFZ and Obato to mice inoculated with SUDHL4 cells substantially suppressed tumor growth, activated JNK, inactivated AKT, and increased survival compared to the effects of single agent treatment. Together, these findings argue that a strategy combining CFZ and Obato warrants attention in DLBCL.
INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) accounts for ~ 30% of all non-Hodgkin’s 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 25-30% 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 sub-types, i.e., 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 (MM) and mantle cell lymphoma (MCL) (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 sub-types (7). Consequently, development of strategies active against both GC and ABC subtypes remains the subject of major interest. Carfilzomib, an irreversible proteasome inhibitor, exerts pre-clinical 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) (10) (11), ER stress (11), activation of the stress-related JNK (c-Jun N-terminal kinase) pathway (12), and inhibition of NF-κB-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 anti-apoptotic 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 (oblatoclax), 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 demonstrated synergistic interactions between proteasome inhibitors (e.g., bortezomib) and BH3-mimetics (e.g., HA14-1) in malignant hematopoietic cells, including multiple myeloma, mantle cell lymphoma 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 ABC- and GC-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 sub-types and in bortezomib-resistant cells. Furthermore, potentiation of carfilzomib lethality by obatoclax depends functionally on activation of the stress kinase JNK, induction of Noxa, down-regulation of AKT phosphorylation and release of pro-apoptotic 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.
MATERIAL 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 supplemental Methods (13). SUDHL16-shJNK cells were generated by electroporation (Amaxis, GmbH, Germany) using buffer L as described previously (13). Cells ectopically expressing activated AKT were generated by transfecting pUSE-myr-AKT1 cDNA (Upstate, Lake Placid, NY) into SUDHL16 cells as before (13). Stable clones were selected by serial dilution using antibiotics (13). Five (5) drug resistant clones were selected for each type (sh-JNK and AKT-CA). They validated functionally significance and results employing two or three randomly selected clones are shown. All experiments were performed with logarithmically growing cells (e.g., 4.0-5.0 x 10^5 cells/ml) within passages 6-24 to ensure uniform responses. Mycoplasma tests were uniformly negative (MycoAlert Mycoplasma Detection Kit, Lonza, Inc., Rockland, ME). The construct pcDNA3.1-Mcl-1 was a generous gift from Dr. R.W. Craig (Dartmouth, Hanover NH) and used to express Mcl-1 in SUDHL4 cells by transient transfection. HuSH 29 mer shRNA constructs against NOXA1 in a pRFP-C-RS vector from Origene Technologies, Rockville, MD, (Cat. No. TF311134) were used to knock down NOXA in SUDHL4 cells through transient transfection. Cell lines were authenticated by STR DNA fingerprinting using the AmpFISTR Identifiler kit (Applied Biosystems). The STR profiles were compared with known American Type Culture Collection (ATCC) data base and to the German Collection of Microorganisms and Cell Cultures database (http://www.dsmz.de/).

Transient transfection

Transient transfection of SUDHL4 cells employed an Amaxis Nucleofector shuttle apparatus (Cologne, Germany) as per protocol in 96 well plate mode (details in Supplementary Methods).
Reagents

Carfilzomib was provided by Onyx Pharmaceuticals, Emeryville, CA. Bortezomib (Velcade) was from Millennium Pharmaceuticals, Cambridge, MA. Obatoclax (formerly GX15-070) was from Cephalon, Frazer, PA. 7-Aminoactinomycin D (7-AAD) was purchased from Sigma-Aldrich, St. Louis, MO. All agents were formulated in DMSO. Structures of carfilzomib and obatoclax were illustrated in Supplementary Fig. 1

Experimental Format

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

Assessment of cell death and apoptosis

Cell viability was monitored by flow cytometry using 7-amino actinomycin D 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 (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: pAKT, AKT1, p-JNK, JNK1, p-p44/42, p44/42, BID, Bcl-xL were from Santa Cruz Biotechnology, Santa Cruz, CA.; cleaved caspase-3, P-histone-H2AX were from Cell Signaling Technology, Beverly, MA; PARP (C-2–10)was from Upstate Biotechnology, Lake Placid, NY; Tubulin was from Oncogene, San Diego, CA. Actin antibodies were purchased from Sigma, St. Louis, MO. Bcl-2 antibodies were from Dako, Carpinteria, CA. Mcl-1 antibodies were purchased from BD BioScience, Sparks, MD.
Immunoprecipitation

Analyses of protein complexes by immunoprecipitation including conformationally changed Bak were performed using CHAPS lysis buffer and immunomagnetic Dynabeads M-450 microspheres (Invitrogen) (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 oligomerisation was studied as described in Supplementary Methods.

Animal Studies

Animal studies were performed utilizing Beige-nude-XID mice (NIH-III; Charles River, Wilmington, MA, USA). 10x10^6 SUDHL4 cells were pelleted, washed twice with 1X PBS, and injected subcutaneously into the right flank. Once the tumors were visible, mice were grouped into four separate sets with 5 to 6 mice in each set. The control group was treated with vehicle alone (10mM citrate buffer and 5% dextrose solution) and the remaining three groups were treated with carfilzomib ± obatoclax. Carfilzomib was administered via tail vein (IV BIW) injection BIW (days 1,2, 8,9, 15, 16 etc.), and vorinostat was administered IP TIW (days 1,2,3, 8,9,10 etc.). Tumor volume was measured 2-3 times per week with calipers using the following formula: Tumor Volume (mm^3) = Length (mm) X Width (mm) (13). Tumor infiltration was monitored by bioluminescence imaging once or twice a week. Once the tumor size reached 2000 mm^3, mice were sacrificed. Survival was evaluated from the first day of treatment until death using Kaplan–Meier curves. All experiments were performed in triplicate. Details of drug formulation are in Supplementary Methods.
Statistical Analysis

Differences between experimental conditions were assessed using two-sided 0.05 level t-tests. Synergistic drug interactions were formally tested using Laska’s model-free (27, 28) test for synergy, implemented with unequal-variance 0.05 level two-sample t-tests in R, and characterized via contour plots of the dose-response surface. Synergism was also evaluated by median dose effect analysis using a software program (CalcuSyn, Biosoft, Ferguson, MO) (29). Cells were treated with carfilzomib and obatoclax at fixed concentration ratios (1:375) for 48 hrs and cell death was monitored by 7AAD staining and flow cytometry. Combination index (C.I.) values and isobolograms were computed using CalcuSyn software. Combination index values less than 1.0 denote synergism. Survival functions were estimated using Kaplan-Meier curves and compared using the logrank test.
RESULTS

Carfilzomib and obatoclax interact synergistically to induce apoptosis in GC- and ABC-DLBCL cells

Whereas exposure (24 hr) to 150-250 nM obatoclax alone minimally induced apoptosis in GC-DLBCL SUDHL-16 cells, co-exposure to carfilzomib concentrations as low as 1.5 nM significantly increased apoptosis, and concentrations $\geq$ 3.5 nM resulted in apoptosis in the large majority of cells (Fig 1A). Conversely, 2-3 nM carfilzomib by itself was minimally toxic, but co-administration of obatoclax concentrations as low as 100-200 nM significantly increased cell death, and concentrations $\geq$ 250 nM 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 hr, increasing further over the ensuing 48 hr (Figure 1C). Two approaches (Laska’s model-free test and Median Dose Effect analysis) were used to evaluate synergism. For Laska’s test, (56) several dose combinations of carfilzomib and obatoclax with non-zero 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 Laska’s 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 < 0.0001 to 0.0027 (Fig 1D). Median Dose Effect analysis revealed Combination Index values $<$ 1.0, indicating synergistic interactions (Fig 1E). Based on this analysis, mean CFZ (2.5nM) and obatoclax (200nM) concentrations were employed for subsequent mechanistic studies. Similar synergistic interactions were observed in SUDHL-4 (Supplementary Fig 2) 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 sub-type) and OCI-LY3 (ABC sub-type) cells (Fig 1F). Finally, significant increases in lethality were observed when primary DLBCL cells (one GC- and one ABC- sub type) cells were co-exposed to carfilzomib and obatoclax (Fig 1G). However, co-exposure to carfilzomib and obatoclax did not significantly potentiate lethality compared to single agent treatment in normal CD34$^+$ bone marrow cells (Fig 1H).
Carfilzomib/obatoclax exposure activates JNK, inactivates AKT, up-regulates Noxa, and induces γH2A.X in DLBCL cells

Exposure of SUDHL16 cells to carfilzomib (24 hr; 2.5 nM) or obatoclax (200 nM) alone minimally induced caspase-3 cleavage and PARP degradation (Fig 2A). In contrast, co-administration 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 SUDHL-4 cells (data not shown). Combined treatment also reduced expression of phospho-AKT but not total AKT expression (Fig 2C). In contrast, obatoclax increased phospho-ERK1/2, an effect that was attenuated by carfilzomib-co-exposure.

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 bortezomib (24, 30), carfilzomib modestly but discernibly increased Mcl-1 levels (Fig 2E). Time course analysis demonstrated an increase in Mcl-1 levels appreciable after 6-hr exposure to 2.5 nM carfilzomib, and pronounced at intervals ≥ 12 hr (Fig 2E). Notably, obatoclax sharply decreased Mcl-1 expression and attenuated carfilzomib-mediated down-regulation. 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 nM; 24 hr) diminished NF-κB activity in both SUDHL-16 and OCI-LY10 cells by approximately 30-40% but this effect was not enhanced by obatoclax (Supplementary Fig 3).

In view of evidence that obatoclax triggers autophagy in malignant hematopoietic cells (33), the effects on autophagy were examined in SUDHL-16 cells. Obatoclax (200 nM) 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 hrs) of SUDHL-16 cells to carfilzomib (2.5 nM) or 200nM obatoclax individually triggered Bax mitochondrial translocation, combined treatment resulted in a very
pronounced increase (Supplementary Fig 4A). Consistent with previous reports, Bak was localized to the mitochondria (34) and levels increased modestly following obatoclax ± carfilzomib exposure (Supplementary Fig 4A). Carfilzomib and to a lesser extent obatoclax triggered Bax conformational change/activation, whereas combined treatment induced a marked increase (Supplementary Fig 4B). 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 4C).

Immunoprecipitation studies revealed that obatoclax but not carfilzomib diminished Mcl-1/ Bim binding, whereas combined treatment dramatically reduced this association (Supplementary Fig 4D). Moreover, obatoclax ± carfilzomib sharply diminished the Mcl-1/Bak association (Supplementary Fig 4D). 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 4E).

*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, SUDHL-16 cells were stably transfected with scrambled sequence or JNK shRNA constructs (CL8 or CL12). Antisense clones displayed a partial but clearly discernible reduction in JNK expression compared to 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 phospho-JNK induction and caspase-3 cleavage in CL8 clones compared to controls (Fig 3B).

Parallel studies were performed using SUDHL-16 cells ectopically expressing constitutively active AKT. Two clones (AKT cL3 and cL5) displayed significantly increased phosphorylation
of the AKT target GSKα/β compared to empty-vector controls (Fig 3C, upper panel) and exhibited partial but significant (P < 0.05) reductions in carfilzomib/obatoclax lethality compared to controls (Fig 3C, lower panel). They also displayed diminished inhibition of AKT phosphorylation, PARP cleavage, and caspase-3 activation compared to 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.

Noxa up-regulation and Mcl-1 down-regulation play functional roles in carfilzomib/obatoclax lethality in DLBCL cells

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

Parallel studies were performed with SUDHL-4 cells ectopically expressing Mcl-1 by transient transfection. As shown in Fig 4C (upper panel), Mcl-1 was overexpressed following transfection of pcDNA3.1-Mcl-1 cDNA versus empty vector control pcDNA3.1 (Fig 4C, upper panel), associated with a significant reduction in apoptosis following carfilzomib/obatoclax exposure (P < 0.05; Fig 4C, lower panel). Notably, carfilzomib/obatoclax-treated Mcl-1 overexpressing cells displayed a marked increase in Mcl-1 co-immunoprecipitating with Bim compared to empty vector controls (Fig 4D).

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 employed (13, 20).
These cells exhibit no lethality in the presence of 10, or 40 nM bortezomib respectively, whereas essentially 100% of parental cells die under these conditions. Co-administration of carfilzomib (concentrations 5-20nM) and obatoclax (concentrations 100-250nM), which were minimally toxic by themselves, sharply increased lethality when co-administered (Fig 5A). Combination Index 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 up-regulation and increased $\gamma$H2A.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 co-immunoprecipitating with Bim and Bak, and diminished co-immunoprecipitation of Bcl-xL with Bak compared to 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 SUDHL-4 xenograft model was employed (13). While 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 analysis also demonstrated that that carfilzomib significantly increased the survival of obatoclax-treated mice (P < 0.05; Fig 6C). Moreover, Western blot analysis of tumor sections revealed combined carfilzomib/obatoclax exposure clearly increased phospho-JNK expression and reduced expression phospho-AKT expression compared to single agent treatment, as observed in vitro ( Fig 6D). Finally, combined treatment resulted in minimal weight loss and only minor reductions in white blood cell counts (Supplementary Fig 5), indicating that combined carfilzomib/obatoclax treatment is tolerable in intact animals and recapitulates at least some of the effects seen in DLBCL cells in vitro.
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 anti-apoptotic 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’s lymphoma models (20). The present studies were prompted by several considerations. First, single-agent activity of bortezomib in DLBCL is limited (6) and demonstrated 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 e.g., ABT-737 (16), down-regulates 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 demonstrate that a dual approach involving the second-generation proteasome inhibitor carfilzomib and the pan-BH3 inhibitor obatoclax is effective against ABC- and GC-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 up-regulation, dissociation of Bcl-2/Bcl-xL/Mcl-1 from pro-apoptotic effectors) contribute to synergistic interactions.

While proteasome inhibitors trigger accumulation of pro-apoptotic proteins (e.g., Bim), they may also up-regulate anti-apoptotic 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 up-regulation of Noxa (38), a protein capable of down-regulating Mcl-1 (30), potentiates bortezomib lethality in multiple myeloma, mantle cell lymphoma, and other models (17) (19, 20). Here, carfilzomib alone up-regulated Mcl-1, an event prevented by obatoclax co-
administration. 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 mantle cell lymphoma cells (38), obatoclax promoted Mcl-1/Bak dissociation in DLBCL cells. However, this phenomenon was more marked with obatoclax/carfilzomb co-exposure, and occurred at considerably lower obatoclax concentrations than previously reported e.g., ~200 nM versus $\geq 1 \mu M$, possibly reflecting the pronounced Mcl-1 down-regulation 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 proteasome inhibitors lethality in myeloma cells (30), and Noxa induction contributes significantly to obatoclax/bortezomib interactions in mantle cell lymphoma 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 events e.g., up-regulation of Noxa, un-tethering 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 over-expression or Noxa knock down diminished carfilzomib/obatoclax-induced apoptosis argues that perturbations in these proteins contribute to the enhanced lethality.

The MAP kinase JNK is activated by diverse stresses, and generally exerts pro-apoptotic effects, in contrast to ERK1/2, 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 $\lambda$H2A.X expression, reflecting double-stranded DNA breaks (31). In this context, proteasome inhibition interferes with DNA repair processes (31, 42), and mantle cell lymphoma 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 knock down 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. 6

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 up-regulation or mutation of proteasome sub-units, induction of anti-oxidative defenses, or up-regulation of anti-apoptotic proteins such as Mcl-1, among others (46). Although carfilzomib exhibits significant activity against certain bortezomib-resistant cells (e.g., myeloma) (8, 13), cross-resistance i.e., in DLBCL or mantle cell lymphoma 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 nM) concentrations of carfilzomib. Significantly, several mechanisms implicated in carfilzomib/obatoclax interactions in bortezomib-sensitive cells e.g., 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 co-administration in immunodeficient mice inoculated with DLBCL cells sharply reduced tumor cell growth accompanied by increased survival compared to single agent treatment. Furthermore, several of the events implicated in in vitro synergism e.g., phospho-JNK up-regulation and phospho-AKT down-regulation 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 up-regulation, JNK activation, AKT inactivation, λH2A.X induction) could serve as correlative response determinants in future clinical trials involving 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 demonstrated 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.
Acknowledgments: These studies were supported by Lymphoma SPORE award 1P50 CA130805.

Contribution: G.D. - designed and performed the research, collected, analyzed, and compiled data, and helped to write the manuscript; D.L., M.S., H.L., E.A. - performed the research, collected, analyzed and compiled data; D.P. - perform statistical analysis of the experimental data; P.D., R.F., J.F. - helped to design the research and write the manuscript; S.G. - supervised the research, and helped to write the manuscript.

Financial support: SG, GD, and DL were supported by awards CA63753, CA93738, and CA100866 from the National Institutes of Health; award R6059-06 from the Leukemia and Lymphoma Society of America, the Multiple Myeloma Research Foundation, Myeloma Spore (P50CA142509) and the V Foundation. SG, GD, DL, MS, RF, and JF were supported by Lymphoma SPORE award 1P50 CA130805.
Reference List


Pei XY, Dai Y, Grant S. The proteasome inhibitor bortezomib promotes mitochondrial injury and apoptosis induced by the small molecule Bcl-2 inhibitor HA14-1 in multiple myeloma cells. Leukemia 2003;17:2036-45.


LEGENDS

Figure 1  Carfilzomib/obatoclax co-treatment synergistically induces cell death in DLBCL lymphoma lines and primary lymphoma cells but not in normal cells

(A). SUDHL16 cells were treated (48 hr) with carfilzomib (CFZ) (2.0 - 4.0 nM) ± obatoclax (obato;150-250 nM), after which apoptosis was monitored by 7AAD. B. SUDHL16 cells were treated (48 hr) with 100-300 nM obatoclax ± carfilzomib (2.0-3.0 nM), followed by 7AAD staining. C. Time course of SUDHL16 cell death (7AAD) following obatoclax/carfilzomib exposure. (D) Synergistic interactions between carfilzomib and obatoclax at various concentrations in SHDHL16 cells were tested using Laska’s model-free test for synergy as in Methods. Synergism was observed for all 8 combinations tested with P values ranging from <0.001 to 0.0027. (E) Combination Index (C.I.) values were determined using CalcuSyn software employing fixed drug ratios of carfilzomib and obatoclax (1:80) in SUDHL16 cells. (F) OCI-LY10 and OCI-LY3 cells were treated (48 hr) with carfilzomib (5.0 and 4.0 nM respectively) ± obatoclax (50 and 75nM respectively); cell death was monitored by 7AAD/DiOC6 staining. (G) Two primary human DLBCL (one ABC and other GC sub-type) specimen was isolated and treated with carfilzomib (2 nM and 100 nM for ABC and GC sub types respectively) ± obatoclax (75nM and 125nM for ABC and GC sub types respectively) for 14 h, after which cell death was monitored by 7AAD staining and normalized to controls. Viability of untreated primary specimens was 80% (H) CD34+ cells were exposed (48 hr) to carfilzomib (10-20 nM) ± obatoclax (500 nM). Cell death was monitored by 7AAD staining and normalized to controls. For all studies, values = means for 3 experiments performed in triplicate ± S.D. For A-C, F, G * = values significantly greater than those for carfilzomib or obatoclax treatment alone; P < 0.01.

Figure 2- Carfilzomib/obatoclax co-exposure activates JNK, inactivates AKT, up-regulates Noxa, and induces γH2A.X in DLBCL cells

SUDHL16 cells were treated (24 hrs) with carfilzomib (2.5 nM) ± obatoclax (200nM). (A-D) Expression of the indicated proteins was determined by Western blotting (E), SUDHL16 cells were treated with 2.5nM of carfilzomib and Mcl-1 determined by Western blotting. Each lane was loaded with 20 µg of protein; blots were stripped and re-probed with antibodies directed against tubulin to ensure equivalent loading and transfer. Results are representative of three
separate experiments (F) OCI-Ly10 cells were treated with carfilzomib (4.0 nM) ± obatoclax (50nM) for 24 hrs and expression of the indicated proteins determined by Western blotting.

Figure 3- Genetic interruption of JNK and AKT significantly diminishes carfilzomib/obatoclax lethality

(A) SUDHL16 - JNK shRNA or scrambled sequence vectors were exposed (48 hr) to carfilzomib (2.5nM) + obatoclax (150nM), after which, cell death was monitored by 7AAD. Inset: expression of JNK protein in SUDHL16-scrambled sequence and shJNK clones. (B) Following 24 hr treatment as in (A) protein was monitored by Western blot (C) SUDHL16 cells were stably transfected with constitutively active (myristolated) AKT constructs (AKT cl.3 and 5) or empty vector (pUSE) and exposed (48 hr) to carfilzomib (3.0nM) + obatoclax (150nM), after which cell death was monitored by 7-AAD. Inset: Western blots showing expression of AKT and p-GSK-3α/β in empty vector control and AKT clones. (D) Cells were treated as described above in (C) for 24 hrs, after which Western blot analysis was performed 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.

Figure 4- Noxa up-regulation and Mcl-1 down-regulation 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 hr) to carfilzomib (1.5 nM) + obatoclax (1.0 µM), after which. Cell death was monitored by 7-AAD. Inset: Western blots showing expression of Noxa in empty vector control and Noxa transfected cells following carfilzomib (1.5 nM) + obatoclax (1.0µM) treatment. (B) Cells were treated as in (A) for 24 hrs, 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 hr) to carfilzomib (2.0 nM) + obatoclax (1.0 µM) for 48 hrs. Cell death was monitored by 7-AAD. Inset: Western blots showing expression of Mcl-1 in empty vector control and transiently transfected cells. (D) Cells were treated as in (C) for 24 hrs, after which Western blot analysis was performed to monitor...
protein expression. Immunoprecipitation was employed to characterize the association between Bim and McI-1. For A and C * = significantly less than values obtained for carfilzomib + obatoclax treatment in SUDHL16-scrambled sequence or SUDHL16 expressing pcDNA3.1 control cells; P < 0.05.

Figure 5- The CFZ/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 hr) with minimally toxic concentrations of carfilzomib and obatoclax. Concentrations were as follows: SUDHL16-10BR - carfilzomib (5 nM) ± obatoclax (250 nM), OCI-LY10-40BR - carfilzomib (20 nM) ± Obatoclax (100 nM); Cell death was monitored by 7AAD. (B) Median Dose Effect analysis yielded. Combination Index (C.I.) values < 1.0 denoting synergistic interactions. C. SUDHL16-10BR cells were exposed (24 hr) to carfilzomib and obatoclax as in (A), after which Western blot analysis was performed.. D. SUDHL16-10BR and OCI-LY10-40BR cells were treated with carfilzomib and obatoclax for 24 hrs as in (A) after which, associations between McI-1 and Bak and BIM, Bcl-xL with Bak were characterized by immunoprecipitation followed by Western blotting.

Figure 6- Obatoclax markedly potentiates CFZ induced tumor growth suppression, survival, JNK activation, and AKT dephosphorylation in an in vivo SUDHL4 xenograft model

NIH-III nude mice were injected in the flank with (A) 10 x10⁶ SUDHL4-luciferase cells and treated with carfilzomib (2.0mg/kg) ± obatoclax (3.0mg/kg) as described in Methods. Tumor volumes were measured twice weekly and mean tumor volumes were plotted against days of treatment. (B) Tumor progression was also monitored using an IVIS bioimager. (C). Survival curve of individual groups (control-blue line, carfilzomib- green line , obatoclax - yellow line, , carfilzomib + obatoclax - purple line) was evaluated from the first day of treatment until death using Kaplan–Meier curves (*, P < 0.05). (D) Tumor samples were lysed with lysis buffer followed by sonication. Western blotting was performed to monitor expression of the indicated proteins.
Figure 1

A. CFZ + Obato 150nM
B. CFZ + Obato 200nM
C. CFZ + Obato 250nM
D. Obatocilax (nM)
E. % Cell Death
F. OBato + CFZ
G. % Apoptotic cells (relative to control)
H. % Cell Death (relative to control)
Figure 2
Figure 3

A

JNK

Tubulin

scram
shJNK CL8
shJNK CL12

% Cell Death

cont CFZ + obato

B

scram
shJNK CL8

p-JNK

CF PARP

CF caspase3

Tubulin

cont CFZ + obato

C

AKT

p-GSK-3α/β

Tubulin

pcDNA3.1
AKT cl.3
AKT cl.5

% Cell Death

cont CFZ + obato

D

pUSE

pUSE-AKT cl. 3

CF PARP

CF caspase3

Tubulin

cont CFZ + obato

* * *
Figure 4
Figure - 5
Figure 6

A) Tumor Volume (mm³)

- cont
- CFZ
- obato
- CFZ+obato

Days

B) Control
CFZ - 2.0mg/kg
Obato – 3.0 mg/kg
CFZ + obato

C) Survival Curve

- Cont
- CFZ
- Obato
- CFZ+Obato

Cum Survival

Days

(P < 0.05)

D) Tumor sample

- p-JNK
- p-AKT
- Tubulin

Figure-6
Molecular Cancer Therapeutics

Obatoclax interacts synergistically with the irreversible proteasome inhibitor carfilzomib in GC- and ABC- DLBCL cells in vitro and in vivo

Girija Dasmahapatra, Dmitry Lembersky, Minkyeong P Son, et al.

Mol Cancer Ther Published OnlineFirst March 12, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0021

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/08/31/1535-7163.MCT-12-0021.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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

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

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