Preclinical Development

Carfilzomib Interacts Synergistically with Histone Deacetylase Inhibitors in Mantle Cell Lymphoma Cells In Vitro and In Vivo

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Abstract

Interactions between the proteasome inhibitor carfilzomib and the histone deacetylase (HDAC) inhibitors vorinostat and SNDX-275 were examined in mantle cell lymphoma (MCL) cells in vitro and in vivo. Co-administration of very low, marginally toxic carfilzomib concentrations (e.g., 3–4 nmol/L) with minimally lethal vorinostat or SNDX-275 concentrations induced sharp increases in mitochondrial injury and apoptosis in multiple MCL cell lines and primary MCL cells. Enhanced lethality was associated with c-jun-NH2-kinase (JNK) 1/2 activation, increased DNA damage (induction of λH2A.X), and ERK1/2 and AKT1/2 inactivation. Co-administration of carfilzomib and histone deacetylase inhibitors (HDACI) induced a marked increase in reactive oxygen species (ROS) generation and C2-M arrest. Significantly, the free radical scavenger tetrakis(4-benzoic acid) porphyrin (TBAP) blocked carfilzomib/HDACI-mediated ROS generation, λH2A.X formation, JNK1/2 activation, and lethality. Genetic (short hairpin RNA) knockdown of JNK1/2 significantly attenuated carfilzomib/HDACI-induced apoptosis, but did not prevent ROS generation or DNA damage. Carfilzomib/HDACI regimens were also active against bortezomib-resistant MCL cells. Finally, carfilzomib/vorinostat co-administration resulted in a pronounced reduction in tumor growth compared with single agent treatment in an MCL xenograft model associated with enhanced apoptosis, λH2A.X formation, and JNK activation. Collectively, these findings suggest that carfilzomib/HDACI regimens warrant attention in MCL. Mol Cancer Ther; 10(9); 1686–97. ©2011 AACR.

Introduction

Mantle cell lymphoma (MCL) is an aggressive form of non–Hodgkin’s lymphoma (NHL) characterized by the translocation t(11;14)(q13;q32) and overexpression of cyclin D1 (1). Other abnormalities include dysregulation of additional cell cycle regulatory proteins such as p15 and p16 and mutations in ataxia-telangiectasia mutated (ATM; ref. 2). Overall survival in MCL is less than 4 years, and generally less than 15% of patients experience long-term survival (3). A cause for optimism in MCL treatment has been the incorporation of targeted agents into the therapeutic armamentarium. For example, the proteasome inhibitor bortezomib has significant single agent activity in MCL and is approved for patients with refractory disease (4). In addition, the alkylating agent-like drug bendamustine, particularly in combination with rituximab, has shown promising activity in MCL (5). Despite these encouraging developments, more effective therapies are clearly needed. For example, in patients with refractory MCL receiving bortezomib, response rates are only 30% and progression-free survival is only 6.5 months (6).

The proteasome plays a critical role in cellular homeostasis, particularly the disposition of misfolded or other unwanted proteins. The catalytic proteasome 20S core exhibits chymotrypsin-like (C-T), trypsin-like (T), and caspase-like (C) activities, which are variably inhibited by proteasome inhibitors (7). Mechanisms of bortezomib lethality are not known with certainty, but have been attributed to multiple actions, including inhibition of nuclear factor kappab (NF-kB) by preventing degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), accumulation of proapoptotic proteins, or induction of oxidative injury (8–10). The preexistence or development of bortezomib resistance has prompted the development of second-generation proteasome inhibitors (11). One such agent, carfilzomib (PR-171), is an epoxyketone that, unlike bortezomib, irreversibly inhibits the 26S proteasome (7, 12). In preclinical studies, carfilzomib has shown...
activity against bortezomib-resistant cells (12) and is currently undergoing clinical evaluation in multiple myeloma and other hematopoietic malignancies (13). Its activity in MCL has not yet been fully evaluated. In preclinical studies, both carfilzomib and bortezomib increase the activity of the BH3-mimetic ABT-737 in MCL cell lines (14).

Histone deacetylase inhibitors (HDACi) represent epigenetic agents that modify chromatin structure through histone tail acetylation, thereby promoting gene expression associated with cell death and/or differentiation (15). However, like proteasome inhibitors, HDACis kill cells through multiple mechanisms, including upregulation of death receptors, induction of oxidative injury, and disruption of DNA repair, among others (16). HDACis have been approved for the treatment of cutaneous T-cell lymphomas (17). They also show activity against MCL cells in preclinical studies (18) and are being evaluated in patients with MCL and other lymphomas (19). Notably, recent profiling studies have shown epigenetic silencing of cell cycle regulatory genes (e.g., p15) in MCL cells, providing a theoretical foundation for using HDACis in this disease (20).

Multiple groups have described synergistic interactions between histone deacetylase (HDAC) and proteasome inhibitors in malignant hematopoietic cells, including MCL. Mechanisms invoked to account for such interactions include interruption of NF-κB, disruption of aggresome function, and induction of endoplasmic reticulum stress (21, 22). Significantly, a regimen combining bortezomib and the pan-HDACi vorinostat exhibited encouraging activity in patients with refractory multiple myeloma, including some who have progressed on bortezomib (23). Recently, our group described synergistic interactions between carfilzomib and vorinostat in diffuse large B-cell lymphoma (DLBCL) cells, including both ABC and GC subtypes in vitro and in vivo (24). Despite certain similarities, the biology, genetic background, and clinical course of MCL and DLBCL differ in multiple respects. The purpose of the present studies was to determine whether HDACis enhanced carfilzomib activity in MCL cells, and if so, by what mechanisms. Our results indicate that carfilzomib and HDACis interact synergistically in MCL cells in vitro and in vivo through a process that involves oxidative injury and activation of the stress-related c-Jun-NH2-kinase (JNK) signaling pathway.

Materials and Methods

Cells
Mantle cells (i.e., Granta 519, Rec-1, HF-4B, JVM-2, MINO, and JVM-13) were provided by Dr. Steven Bernstein, Wilmot Cancer Center, University of Rochester Medical Center, New York. Bortezomib-resistant Granta-281BR were generated by exposing parental cells to increasing bortezomib concentrations starting with 1.0 nmol/L, as described previously in DLBCL cells (24). Granta-JNK short hairpin RNA (shRNA) cells were generated by electroporation (Amaza GmbH) of JNK shRNA cDNA to Granta cells, using buffer C as described (24) to generate SUDHL1-JNK shRNA clones. Four separate sequences were used to knock down JNK1 (i.e., 1-CTGCAACAGCATTTAGATAA, 2-CAGAGACTAGTTCTATGAA, 3-CCTACAGAGCTAGTCTCTA, and 4-CGACGGTTATGCTGATCATC) and 1 nonspecific negative control sequence (NC-GGAATCTCATTCGATGCATC). Granta cell clones with sequence 2 displayed maximal differential expression of JNK1 compared with controls and were used for analysis. Stable clones were selected by serial dilution using G418 as a selection marker (24). Cell lines were authenticated by short tandem repeat DNA fingerprinting using the Applied Biosystems Identifier Kit. The short tandem repeat profiles were compared with the known American Type Culture Collection database and to the German Collection of Microorganisms and Cell Cultures database (http://www.dsmz.de/). Cells were last tested on June 2011.

Reagents
Carfilzomib and its water soluble derivative ONX 0912 were from Onyx Pharmaceuticals. Bortezomib (Velcade) was from Millennium Pharmaceuticals. Vorinostat was from Merck & Co., Inc. SNDX-275 was from Syndax Pharmaceuticals. 7-Aminoactinomycin D (7-AAD) was from Merck & Co., Inc. SNDX-275 was from Syndax Pharmaceuticals. 7-Aminoactinomycin D (7-AAD) was purchased from Sigma-Aldrich. Suberoyl bis-hydroxamic acid (SBHA) was purchased from Biomol. BOC-fmk was purchased from MP Biochemicals. All agents were formulated in dimethyl sulfoxide.

Experimental format
Cells were cultured as described earlier (24). Cells were treated with desired drugs and prepared for analysis as described later.

Assessment of cell death and apoptosis
Cell viability was monitored by flow cytometry using 7-AAD staining as previously described (24). Alternatively, Annexin V/propidium iodide (PI) staining (both BD Pharmingen) was used to monitor early (Annexin V+) or late (Annexin V+, PI+) apoptosis as described before (24). In all studies, results of 7-AAD and Annexin V/PI assays were concordant.

Collection and processing of primary mantle cells
These studies have been approved by the Investigational Review Board of Virginia Commonwealth University. CD34+ cells were isolated using an immunomagnetic bead separation technique as described (24).

Western blot analysis
Western blot samples were prepared from whole cell pellets as described (24). Source of primary antibodies were as follows: phospho (p)-AKT, AKT1, p-JNK, JNK1, p-p44/p42, p44/p42, SOD2, CD38, CD138 (Syndecan-1), and IRF4 were from Santa Cruz Biotechnology; cleaved

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caspase-8, cleaved caspase-3, and p-histone-H2A.X were from Cell Signaling Technology; PARP (C-2-10) and SMAC were from Upstate Biotechnology; caspase-8 was from Alexis; and tubulin was from Oncogene. Actin was purchased from Sigma-Aldrich. Bcl-2 was from Dako.

NF-κB activity
Nuclear protein was extracted using a Nuclear Extract Kit (Active Motif). NF-κB activity was determined by an ELISA TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instructions (24).

Electrophoretic mobility shift assay
A total of 5 μg per nuclear protein condition was subjected to electrophoretic mobility shift assay (EMSA) for NF-κB/DNA binding as described previously (24), using [γ-32P] ATP label.

Cell cycle analysis
Cell cycle distribution was determined by flow cytometry using a commercial software program (Modfit; Becton Dickinson) as per standard protocol.

Measurement of reactive oxygen species production
Cells were treated with 20 μmol/L 2',7'-dichlorodihydrofluorescein diacetate for 30 minutes at 37°C, and fluorescence was monitored by flow cytometry using a fluorescence-activated cell sorting scan and analyzed with Cell Quest software (9, 21).

Quantification of glutathione levels in cells
Glutathione levels in cells were measured using a glutathione assay kit provided by Cayman Chemicals, as per the manufacturer’s instructions.

Animal studies
Animal studies were conducted using Beige-nude-XID mice (NIH-III; Charles River). A total of 10 × 106 Granta 514 cells were pelleted, washed twice with 1× PBS, and injected subcutaneously into the right flank. Once the tumors were visible, 5 to 6 mice were treated with carfilzomib ± vorinostat and progress of tumor growth or regression was monitored as described earlier (24). Vorinostat and carfilzomib stock was dissolved in dimethyl sulfoxide and 10% sulforubutylether β-cyclodextrin in 10 mmol/L citrate buffer pH 3.5, respectively. They were stored at −80°C in small aliquots and diluted before injection, as described earlier (24).

Statistical analysis
The significance of differences between experimental conditions was determined using the 2-tailed Student’s t test. Synergistic and antagonistic interactions were defined using median dose effect analysis in conjunction with a commercially available software program (Calcusyn, Biosoft; ref. 24).

Results
Carfilzomib interacts synergistically with HDACIs in multiple MCL cell lines and primary MCL cells
Administration of carfilzomib alone (48 hours) to Granta cells induced minimal toxicity at concentrations 2.5 nmol/L or less, but moderate lethality occurred with higher concentrations (Fig. 1A). In contrast, 1.5 or 2.0 μmol/L vorinostat was essentially nontoxic to these cells. However, coadministration of carfilzomib at concentrations 3.0 nmol/L or more, with nontoxic concentrations of vorinostat, induced apoptosis in virtually 100% of cells. Similar effects, reflected by 7-AAD uptake and loss of ΔΨm, were observed in REC-1 and HF-4B MCL cells (Fig. 1B) and in JVM-2, MINO, and JVM-13 cells (data not shown). Coadministration of minimally or nontoxic concentrations of the HDACIs SBHA or SNDX-275 with marginally toxic concentrations of carfilzomib (2–5 nmol/L) also sharply increased cell death in JVM-2, MINO, and JVM-13 cells (Fig. 1C). Time course analysis of apoptosis induction by carfilzomib/vorinostat in Granta cells revealed a significant increase in lethality by 16 hours, which became more pronounced at intervals of 30 hours or more (Fig. 1D) and reached a plateau after 60 hours. Median dose effect analysis in Granta cells exposed to carfilzomib and vorinostat or SNDX-275 at a fixed concentration ratio (CFZ:vorinostat—4:1,500 and CFZ:SNRX-275—4:1,000) yielded combination index (CI) values substantially less than 1.0, indicating synergistic interactions (Fig. 1E). Similar results were obtained with other MCL lines (data not shown). Finally, combined carfilzomib/vorinostat exposure (48 hours) resulted in a marked increase in lethality compared with single agents in 3 separate primary MCL specimens (Fig. 1F). As previously reported (24), equivalent exposures exhibited minimal toxicity toward normal CD34+ bone marrow cells (data not shown). Finally, combined exposure to HDACIs (vorinostat, valproic acid, or SNDX-275) sharply increased apoptosis by ONX 0912, a water soluble carfilzomib analogue, in multiple MCL cell lines (Supplementary Fig. S1A and B). Structures of carfilzomib, vorinostat, SBHA, SNDX-275, and ONX 0912 are presented in Supplementary Fig. S2.

Combined exposure of MCL cells to carfilzomib and vorinostat induces JNK and caspase activation, MnSOD2 and AH2A.X expression, accompanied by AKT and ERK1/2 phosphorylation
Consistent with increased apoptosis (Fig. 1), combined exposure of HF-4B cells to carfilzomib and vorinostatin sharply increased PARP degradation and cleavage of caspases-3 and -8 (Fig. 2A). As in the case of DLBCL cells (24), combined carfilzomib/vorinostat exposure markedly increased phosphorylation of the stress-related kinase JNK (Fig. 2A), accompanied by dephosphorylation...
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Figure 1. Cotreatment with carfilzomib and HDACIs leads to synergistic induction of cell death in various MCL lines and primary MCL cells. A, Granta cells were treated with varying carfilzomib (CFZ; 1.0–4.0 nmol/L) and with fixed vorinostat (VOR; 1.5 or 0.20 µmol/L) concentrations for 48 hours, after which apoptosis was monitored by Annexin V/PI staining. *, P < 0.01 values significantly greater than those obtained with carfilzomib or vorinostat treatment alone. B, REC-1 and HF-4B cells were treated with varying vorinostat (1.0–2.0 µmol/L) concentrations and fixed carfilzomib concentrations (2.5 or 3.0 nmol/L, respectively) for 36 hours, after which cell death was monitored by 7-AAD/DiOC6 staining. *, P < 0.01 values significantly greater than those obtained with carfilzomib or vorinostat treatment alone. C, JVM-2, NIMO, and JVM-13 cells treated with minimally toxic carfilzomib concentrations (JVM-2, 4 nmol/L; NIMO, 5 nmol/L; and JVM-13, 4 nmol/L) ± SBHA (JVM-2, 30 μmol/L; NIMO, 50 μmol/L; and JVM-13, 40 μmol/L) and SNDX-275 (JVM-2 and NIMO, 1.0 μmol/L; JVM-13, 1.5, μmol/L) for 48 hours, after which cell death was monitored by 7-AAD and DiOC6 staining. **, P < 0.01 values significantly greater than those obtained with carfilzomib or vorinostat treatment alone. D, Granta cells were treated with the indicated concentration of carfilzomib and vorinostat, and cell death at various intervals up to 72 hours was monitored by 7-AAD/DiOC6 staining. E, CI values were determined using CalcuSyn software per the program instruction (24). F, primary human MCL cells were isolated as described in Materials and Methods. They were treated with carfilzomib (first sample, 1.5 nmol/L; second sample, 15 nmol/L; and third sample, 5 nmol/L) ± vorinostat (first sample, 0.75 µmol/L; second sample, 1.25 µmol/L; and third sample, 0.5 µmol/L) for 14 hours. The percentage of apoptotic cells was monitored by Annexin V/PI staining, and the percentage of dead cells was normalized to controls. Viability of the 3 primary specimens without treatment was 75% to 80%, 85% to 75%, and 60% to 70% for 3 samples, respectively. *, P < 0.01 values significantly greater than those obtained with carfilzomib or vorinostat treatment alone. For all studies, values represent the means for 3 experiments carried out in triplicate ± SD.

of ERK1/2 and AKT1/2 without changes in total ERK1/2 or AKT1/2 (Fig. 2B). Notably, vorinostat alone induced SOD2, as previously reported (25), but this was enhanced by carfilzomib (Fig. 2B). In addition, although individual agents minimally induced γH2AX, a double-stranded DNA break marker (26), combined treatment resulted in a pronounced increase (Fig. 2B). Granta cells exposed to the combination of carfilzomib and vorinostat responded similarly (i.e., with enhanced PARP and caspase-3 cleavage, induction of p-JNK, γH2AX, and SOD2, accompanied by AKT1/2 and ERK1/2 dephosphorylation; Fig. 2C). Coadministration of vorinostat and ONX 0912 in HF-4B induced comparable changes (Fig. 2D). Together, these findings indicate that coadministration of carfilzomib and vorinostat leads to a shift away from antiapoptotic (e.g., AKT1/2 and ERK1/2) and toward proapoptotic (e.g., JNK) pathways, accompanied by caspase cleavage, PARP degradation, and various oxidative injury/DNA damage responses (e.g., induction of SOD2 and γH2AX). To determine whether alterations in these signaling proteins represented primary or secondary events, the pan-caspase inhibitor BOC-fmk was used. As shown in Fig. 2E, JNK activation and inhibition of p-ERK1/2 in carfilzomib/vorinostat-treated cells were not diminished by BOC-fmk pretreatment, whereas p-AKT downregulation and DNA damage induction (reflected by γH2AX upregulation) were largely prevented (Fig. 2E). Time course analysis revealed that JNK activation occurred as early as 12 hours after treatment, whereas downregulation of p-ERK and upregulation of γH2AX became apparent at 18 hours and reached maximum level by 24 hours. In contrast, diminished AKT phosphorylation occurred at a relatively late interval (i.e., 24 hours; Supplementary Fig. S3). Together,
these findings suggest that JNK activation and ERK1/2 inactivation represent primary events, whereas AKT inactivation and DNA damage represent secondary events in carfilzomib/vorinostat lethality. Finally, electrophoretic mobility shift assay analysis revealed that carfilzomib coadministration abrogated NF-κB activity assays (Supplementary Fig. S4A). Similar results were observed with ELISA-based NF-κB activity assays (Supplementary Fig. S4B).

Evidence of a role for oxidative injury in carfilzomib/vorinostat lethality in MCL cells

Previous studies have implicated oxidative injury in HDAC/proteasome inhibitor lethality in myeloid and lymphoid malignancies (21, 27). Consequently, HF-4B cells were exposed to carfilzomib ± vorinostat (24 hours) with or without the SOD2 mimetic tetraakis(4-benzoic acid) porphyrin (TBAP), after which reactive oxygen species (ROS) generation was monitored. Significantly, coadministration of carfilzomib and vorinostat, which individually had little effect, markedly increased ROS (>70% over control; Fig. 3A), an effect largely abrogated by TBAP. Enhanced ROS generation was observed after 5 hours, which increased over the ensuing 24 hours (Fig. 3B). Importantly, coadministration of TBAP significantly reduced carfilzomib/vorinostat lethality (P < 0.01; Fig. 3C). TBAP also substantially diminished carfilzomib/vorinostat-mediated PARP and caspase-3 cleavage, JNK activation, and γH2A.X formation (Fig. 3D). In contrast to buthionine sulfoximine (BSO), combined carfilzomib/vorinostat exposure did not reduce glutathione (GSH) levels in HF-4B cells (Fig. 3E). Similar findings were observed in Granta and other MCL cells (data not shown), suggesting a functional role for oxidative injury in carfilzomib/vorinostat lethality.

JNK activation plays a significant functional role in carfilzomib/vorinostat lethality in MCL cells

To assess the functional role of JNK activation in carfilzomib/vorinostat-induced MCL cell death, studies were conducted with Granta cells in which JNK was knocked down by stable expression of JNK shRNA. Three clones, designated C12/11, C14/4, and C14/7, displayed a pronounced reduction in basal JNK expression (Fig. 4A) compared with scrambled sequence controls (Fig. 4A, inset) or untransfected cells (data not shown). JNK shRNA clones were significantly less sensitive than controls to carfilzomib/vorinostat lethality (Fig. 4B). In contrast, the increase in ROS generation following combined carfilzomib/vorinostat treatment was equivalent...
in scrambled sequence and JNK shRNA cells (Fig. 4C), suggesting that ROS generation by this regimen occurs upstream of JNK activation.

**Combined carfilzomib/vorinostat exposure induces G2–M arrest**

Exposure of parental Granta cells to subtoxic concentrations of vorinostat (24 hours) resulted in little change in the cell cycle profile, whereas carfilzomib modestly induced G2–M arrest (Supplementary Fig. S5). In contrast, coadministration of carfilzomib and vorinostat (24 hours) resulted in a very pronounced accumulation of cells in G2–M (e.g., 41%). Similar results were observed in bortezomib-resistant Granta-25BR cells, where combined treatment induced accumulation of 50% of cells in G2–M (data not shown). Accumulation of G2–M cells following carfilzomib/vorinostat exposure was roughly equivalent in scrambled sequence versus JNK shRNA Granta cells and in other MCL lines (e.g., HF-4B; data not shown).

**HDACIs markedly increase carfilzomib lethality in bortezomib-resistant MCL cells**

Parallel studies were conducted in Granta cells cultured in progressively higher concentrations of bortezomib (Granta-25BR), which undergo minimal apoptosis in the presence of bortezomib concentrations of 20 nmol/L (Fig. 5A). Granta-25BR cells also displayed some cross-resistance to carfilzomib concentrations of 10 to 15 nmol/L, although in marked contrast to bortezomib, extensive apoptosis occurred in response to 20 nmol/L carfilzomib (Fig. 5B). Notably, coadministration of minimally toxic concentrations of carfilzomib (e.g., 10 nmol/L) and vorinostat resulted in pronounced apoptosis in Granta-25BR cells (Fig. 5C), accompanied by marked increases in PARP cleavage, JNK activation, γH2A.X formation, as well as diminished ERK1/2 and AKT1/2 phosphorylation (Fig. 5D). A high concentration of bortezomib (e.g., 30 nmol/L), when combined with vorinostat, also effectively killed bortezomib-resistant MCL cells.
Granta cells (Fig. 5C). In addition, ROS generation in Granta-25BR cells increased markedly with carfilzomib/vorinostat treatment, an effect substantially attenuated by TBAP (Fig. 5E). TBAP also significantly attenuated carfilzomib/vorinostat lethality in these cells (Fig. 5F). The time course and caspase dependence of carfilzomib/vorinostat responses were investigated in bortezomib-resistant Granta-25BR cells. Consistent with findings in parental cells, JNK activation occurred before pronounced apoptosis, whereas other events occurred either concurrently (e.g., ERK1/2 inactivation and γH2A.X induction) or subsequently (e.g., AKT inactivation; Supplementary Fig. S6A) with cell death. Moreover, as in parental cells, JNK activation and ERK1/2 inactivation were caspase independent, whereas AKT inactivation and upregulation of γH2A.X were caspase dependent (Supplementary Fig. S6B).

In view of evidence linking bortezomib resistance to plasmacytic MCL differentiation (28), effects of cotreatment with carfilzomib and vorinostat on relevant protein markers (e.g., CD38, CD138, and IRF4) were examined in Granta cells. Significant downregulation of IRF4 was observed following vorinostat ± carfilzomib treatment (Supplementary Fig. S7A), but no changes in CD38 or CD138 expression. Basal levels of these markers were equivalent in parental and bortezomib-resistant Granta-25BR cells (Supplementary Fig. S7B).

**In vivo activity of the carfilzomib/vorinostat regimen in an in vivo Granta xenograft model**

To assess the in vivo activity of the carfilzomib/vorinostat regimen, a Granta-luciferase cell xenograft flank model was used, analogous to the DLBCL model we have described (24). Animals were inoculated in the flank with $10^6$ cells, and following the appearance of tumors, animals were treated with 2.0 mg/kg carfilzomib (i.v., biweekly—days 1 and 2), and 70 mg/kg vorinostat (i.p., twice weekly—days 1, 2, and 3), after which tumor size was monitored twice weekly. Values represent the results of 2 separate experiments carried out independently, and mean tumor volumes for each group were calculated by combining tumor growth data for the 2 experiments. As shown in Fig. 6A, vorinostat alone had minimal effects, whereas carfilzomib moderately reduced tumor growth by day 20. However, vorinostat/carfilzomib coadministration virtually abrogated tumor growth. Parallel studies were conducted in animals inoculated with luciferase-expressing cells, and tumor progression was monitored by a Xenogen IVIS bioimager. Combined treatment resulted in a pronounced reduction in bioluminescence compared with animals treated with single agents or controls (Fig. 6B). Toxicity of combined treatment, for example, hair loss and weight reduction (<10%), was minimal (Fig. 6C). Finally, Western blot analysis obtained from proteins extracted from excised tumors revealed a clear increase in p-JNK, γH2A.X, and cleaved caspase-3 in tumors obtained from animals treated with both agents.
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Figure 5. Bortezomib (BRTZ)-resistant Granta-25BR cells partially cross-resistant to carfilzomib (CFZ) remain sensitive to carfilzomib/vorinostat (VOR)-mediated ROS generation and lethality. Granta and Granta-25BR cells were treated with the indicated concentration of bortezomib (A) and carfilzomib (B) for 48 hours, after which cell death was monitored, as described in Materials and Methods. For B, *, P < 0.01—0.05 significantly less than values obtained for cells treated with carfilzomib + vorinostat. E, Granta-25BR cells were treated with carfilzomib (7.5 nmol/L) ± vorinostat (1.5 μmol/L) for 24 hours, and Western blot analysis was then carried out using the indicated antibodies. E, Granta-25BR cells were treated with the indicated concentration of carfilzomib or bortezomib ± vorinostat for 48 hours, and cell death was monitored by Annexin V/PI. *, P < 0.01 significantly greater than values obtained for cells treated with carfilzomib, bortezomib, or vorinostat alone. D, Granta-25BR cells were treated with carfilzomib (7.5 nmol/L) ± vorinostat (1.5 μmol/L) for 24 hours, and Western blot analysis was then carried out using the indicated antibodies. E, Granta-25BR cells were treated with the indicated concentration of carfilzomib ± vorinostat for 48 hours, and cell death determined by Annexin V/PI. **, P < 0.02 significantly greater than values obtained for cells treated with carfilzomib or vorinostat alone; *, P < 0.05 significantly less than values obtained for cells treated with carfilzomib + vorinostat. F, Granta-25BR cells were treated with the indicated concentration of carfilzomib ± vorinostat for 48 hours, and cell death determined by Annexin V/PI. **, P < 0.02 significantly greater than values obtained for cells treated with carfilzomib or vorinostat alone; *, P < 0.05 significantly less than values obtained for cells treated with carfilzomib + vorinostat.

compared with single agents or controls (Fig. 6D), consistent with in vitro results.

Discussion

The results of this study indicate that HDACIs markedly potentiate the activity of the irreversible proteasome inhibitor carfilzomib in MCL lymphoma cells, including primary MCL cells, as well as MCL cells resistant to bortezomib. Preclinical studies have shown the effectiveness of this strategy in DLBCL, including both the GC and ABC DLBCL subtypes (24), which exhibit disparate gene expression profiles and clinical outcomes (29, 30). Although MCL represents a subtype of NHL, it exhibits several unique features distinguishing it from DLBCL, including the characteristic t(11;14)(q13;q32) translocation, abnormalities in cyclin D and related genes, and perturbations in the DNA damage response (e.g., ATM and p53; refs. 1, 2). Despite the introduction of new agents in this disease, for example, bortezomib (4) and more recently bendamustine (5), MCL clinical outcomes are generally worse than those of DLBCL, with only a minority of patients (e.g., 10%–15%) achieving long-term remissions (1, 3, 31). Consequently, new approaches to MCL treatment are urgently needed. One approach under investigation in NHL involves combining proteasome inhibitors with conventional cytotoxic agents (32) or with other targeted agents, that is, rituximab (33).
The present results suggest that despite differences in molecular pathogenesis, biological features, and clinical behavior, MCL cells, like DLBCL cells, are susceptible to a strategy combining HDACIs with an irreversible proteasome inhibitor such as carfilzomib.

Evidence suggests that oxidative injury plays a significant functional role in carfilzomib/HDACI lethality in MCL cells. Previous studies in both hematopoietic [21, 27] and nonhematopoietic cells [9] have implicated oxidative injury, manifest by increased ROS, in bortezomib lethality. Furthermore, HDACI toxicity toward diverse transformed cell types, including leukemia and lymphoma, has also been attributed to ROS generation [21, 26]. In accord with these findings, combined HDAC and proteasome inhibitor administration triggers a pronounced increase in ROS in leukemia, lymphoma, and myeloma cells [21, 27, 34]. The observation that the antioxidant TBAP significantly reduced carfilzomib/HDACI ROS generation and lethality argues that oxidative injury plays an important functional role in the
synergistic interaction between these agents in MCL cells. The mechanism by which HDAC and proteasome inhibitors interact to potentiate oxidative injury is uncertain, but it may be relevant that both HDAC (35) and proteasome inhibitors (36) induce ROS in transformed cells. The finding that similar events occurred in bortezomib-resistant MCL cells suggests that such cells remain vulnerable to strategies that trigger oxidative damage.

It is noteworthy that coadministration of carfilzomib and vorinostat induced a marked increase in G2–M arrest in MCL cells. This most likely reflects the pronounced increase in DNA damage following carfilzomib/HDACI exposure, manifested by a sharp increase in expression of γH2A.X, an indicator of double-stranded DNA breaks (37). This presumably stems from oxidative injury induced by carfilzomib/HDACI coadministration, as γH2A.X induction was blocked by an antioxidant. Induction of DNA damage characteristically triggers a checkpoint response leading to cell cycle arrest (i.e., in G2–M), which allows cells to undergo repair if the damage is not too extensive or apoptosis if damage is severe (38). It is noteworthy that MCL cells typically exhibit abnormalities in components of DNA damage checkpoints (e.g., ATM and p53; ref. 2), which may contribute to the enhanced apoptotic response. In addition, the ability of HDACIs to disrupt checkpoint responses in transformed cells (39), an important effector in the DNA damage response (40), could amplify cell death in this setting.

Activation of the JNK pathway has been implicated in transformed cell death induced by diverse noxious stimuli, particularly oxidative stress (41). Indeed, JNK activation has been shown to play a functional role in synergistic interactions between HDAC and IκB kinase inhibitors in human leukemia cells (42) and between HDACIs and carfilzomib in DLBCL cells (24). In addition, cross-talk between NF-xB activation and the ROS-dependent activation of the JNK pathway has been extensively described (43). Consistent with this model, the carfilzomib/vorinostat regimen abrogated NF-xB activation and sharply increased both ROS generation and JNK activation. However, the findings that JNK phosphorylation/activation occurs as early as 12 hours after treatment (before the onset of PARP cleavage), that this event is not blocked by caspase inhibitors, and evidence that genetic interruption of JNK function significantly attenuates carfilzomib/vorinostat lethality, support a functional role for JNK activation in cell death. The findings that TBAP blocked JNK activation whereas interruption of JNK signaling failed to diminish ROS generation establishes a hierarchy wherein oxidative injury plays an initiating role and JNK activation represents a downstream effector. The mechanism by which JNK activation leads to cell death remains to be determined but has been attributed to direct mitochondrial injury, possibly mediated by phosphorylation of Bcl-2 family members (44). It may also be relevant that carfilzomib/vorinostat treatment led to inactivation of AKT and ERK1/2, both of which protect malignant hematopoietic cells from oxidative injury (45, 46). However, the relatively late reduction in p-AKT levels by carfilzomib/vorinostat and abrogation of this event by antioxidants argue against a primary role for AKT inactivation in the lethality of this regimen.

Coadministration of carfilzomib and vorinostat, at concentrations and schedules previously shown to be minimally toxic toward normal hematopoietic cells (24), induced pronounced lethality toward both cultured and primary MCL cells. The basis for such in vitro selectivity is uncertain but may be related to the observations that both proteasome (47) and HDAC inhibitors (48), administered individually, display greater toxicity toward transformed versus normal cells. The finding that the carfilzomib/vorinostat regimen effectively inhibited MCL growth while exerting minimal toxicity in an in vivo model reinforces the notion that strategy may preferentially target transformed cells.

In summary, the present results show that regimens combining carfilzomib with HDACIs potently induce apoptosis in MCL cells, including cell lines and primary MCL specimens. They also indicate that oxidative injury and JNK activation play significant functional roles in the lethality of this strategy. Notably, the carfilzomib/vorinostat regimen effectively induced ROS, DNA damage, and apoptosis in bortezomib-resistant MCL cells. Finally, the ability of carfilzomib/vorinostat to inhibit MCL growth in an in vivo xenograft model suggests that this regimen warrants consideration as a therapeutic strategy. Indeed, a phase I trial of vorinostat and carfilzomib in patients with refractory NHL has recently begun. On the basis of the present promising results, patients with MCL will be included in this trial. Apart from defining the maximum tolerated dose for the regimen, it will be of interest to determine whether correlative response determinants in patient samples, for example, levels of p-JNK, can be identified that mimic effects observed preclinically in vitro and in vivo. Such correlative studies are underway to validate the present preclinical observations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

S. Grant, G. Dasmahapatra, and D. Lembersky were supported by awards CA63753, CA93728, and CA100866 from the NIH; award R6509-06 from the Leukemia and Lymphoma Society of America, the Multiple Myeloma Research Foundation, Myeloma Spore (P50 CA142598), and the V Foundation. S. Grant, G. Dasmahapatra, P. Dent, R.I. Fisher, and J.W. Friedberg were supported by Lymphoma SPORE award P50 CA139805. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 15, 2010; revised May 27, 2011; accepted June 27, 2011; published OnlineFirst July 12, 2011.
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Molecular Cancer Therapeutics

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