Small Molecule Therapeutics

In Vitro and In Vivo Interactions between the HDAC6 Inhibitor Ricolinostat (ACY1215) and the Irreversible Proteasome Inhibitor Carfilzomib in Non-Hodgkin Lymphoma Cells

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Abstract

Interactions between the HDAC6 inhibitor ricolinostat (ACY1215) and the irreversible proteasome inhibitor carfilzomib were examined in non-Hodgkin lymphoma (NHL) models, including diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), and double-hit lymphoma cells. Marked in vitro synergism was observed in multiple cell types associated with activation of cellular stress pathways (e.g., JNK1/2, ERK1/2, and p38) accompanied by increases in DNA damage (γH2A.X), G2-M arrest, and the pronounced induction of mitochondrial injury and apoptosis. Combination treatment with carfilzomib and ricolinostat increased reactive oxygen species (ROS), whereas the antioxidant TBAP attenuated DNA damage, JNK activation, and cell death. Similar interactions occurred in bortezomib-resistant and double-hit DLBCL, MCL, and primary DLBCL cells, but not in normal CD34+ cells. However, ricolinostat did not potentiate inhibition of chymotryptic activity by carfilzomib. shRNA knockdown of JNK1 (but not MEK1/2), or pharmacologic inhibition of p38, significantly reduced carfilzomib–ricolinostat lethality, indicating a functional contribution of these stress pathways to apoptosis. Combined exposure to carfilzomib and ricolinostat also markedly downregulated the cargo-loading protein HR23B. Moreover, HR23B knockdown significantly increased carfilzomib- and ricolinostat-mediated lethality, suggesting a role for this event in cell death. Finally, combined in vivo treatment with carfilzomib and ricolinostat was well tolerated and significantly suppressed tumor growth and increased survival in an MCL xenograft model. Collectively, these findings indicate that carfilzomib and ricolinostat interact synergistically in NHL cells through multiple stress-related mechanisms, and suggest that this strategy warrants further consideration in NHL. Mol Cancer Ther; 13(12); 2886–97. ©2014 AACR.

Introduction

Despite recent advances, diffuse large B-cell lymphoma (DLBCL), particularly when relapsed or refractory to initial therapy, remains difficult to treat. Moreover, the activated B-cell (ABC) NF-κB–dependent subtype (1) has a worse prognosis than its germinal center (GC) counterpart (2). Furthermore, double-hit DLBCL with Bcl-2, c-myc, or Bcl-6 upregulation also carries a very poor prognosis (3). Although mantle cell lymphoma (MCL) is characterized as an intermediate-grade lymphoma, it is incurable for most patients (4). Consequently, more effective treatment approaches for these diseases are clearly needed.

Proteasome inhibitors, exemplified by the boronic anhydride bortezomib (Velcade), kill tumor cells through diverse mechanisms, such as upregulation of proapoptotic proteins, induction of reactive oxygen species (ROS) and DNA damage induction, and disruption of protein disposition, etc (5–7). Notably, proteasome inhibitors target neoplastic cells preferentially compared to their normal counterparts (8). Bortezomib has been approved for relapsed refractory multiple myeloma and MCL (9, 10). Its role in DLBCL is less certain, but it may enhance the efficacy of standard chemotherapy in certain DLBCL subtypes (e.g., ABC-DLBCL; ref. 2). Carfilzomib (Kyprolis), an irreversible proteasome inhibitor active in bortezomib-resistant multiple myeloma cells in vitro (11) and in patients with bortezomib-resistant disease (12), is approved for refractory/relapsed multiple myeloma (13). Carfilzomib activity in DLBCL or MCL is less well defined, but multiple trials in these diseases are ongoing.
Histone deacetylase inhibitors (HDACIs) represent epigenetically-acting agents that reciprocally regulate, with histone acetyltransferases (HAT), histone tail acetylation, and, by extension, chromatin structure and gene expression (14, 15). HDACIs are subcategorized on the basis of their selectivity of action, for example, against class I, class IIa/b, or class III HDACs (14). HDACIs kill tumor cells through multiple mechanisms, including death receptor and/or proapoptotic protein upregulation, DNA repair inhibition, and cell-cycle checkpoint disruption, among others (16–18). HDACIs are approved for cutaneous T-cell lymphoma (CTCL)/peripheral T-cell lymphoma (PTCL) and have shown some, albeit limited, single-agent activity in other lymphomas (19). Their main role in the latter diseases may lie in combination strategies (20, 21). Multiple studies have demonstrated synergistic interactions between HDAC and proteasome inhibitors in hematopoietic malignancies (21), particularly multiple myeloma (22, 23). Mechanisms of such interactions are multifactorial, including potentiating of DNA damage, NF-κB inactivation, and aggregatesome disruption (24–26). Recently, attention has focused on development of more selective HDACIs based on the premise that such agents may be more tolerable than pan-HDACIs. One such agent, ricolinostat (ACY1215) is a class IIb tubulin deacetylase inhibitor (27) in clinical development in combination with either bortezomib or lenalidomide to treat relapsed/refractory multiple myeloma (www.clinicaltrials.gov). Notably, ricolinostat displays significant in vitro and in vivo activity in multiple myeloma models, and interacts synergistically with carfilzomib in this setting (28).

Currently, carfilzomib–ricolinostat interactions in non-Hodgkin lymphoma (NHL) systems, including poor prognosis and bortezomib-resistant models, are largely unexplored. Recently, we reported synergistic in vitro and in vivo interactions between carfilzomib and the pan-HDACI vorinostat in DLBCL and MCL cells (21, 29). The purpose of the present studies was to determine whether similar interactions occurred with the more selective HDAC6 inhibitor ricolinostat, and whether such a strategy might be effective in bortezomib-resistant or poor-prognosis subtypes. Our results indicate that ricolinostat interacts synergistically with carfilzomib in multiple DLBCL and MCL systems, including poor-prognosis models, in association with activation of multiple stress- and DNA-damage pathways. Furthermore, this regimen is very well tolerated and active in a murine xenograft MCL model. Collectively, these findings suggest that a strategy combining carfilzomib and ricolinostat warrants attention in relapsed/refractory DLBCL and MCL.

Materials and Methods

Cells

SUDHL4 and OCI-LY7 (all GC-subtype) were obtained from Dr. Liza Rimza (University of Arizona, Tucson, AZ) in December 2006. Granta 519 and Rec-1 (both MCL) were obtained from Dr. Steven Bernstein (James T. Wilmot Cancer Center, Rochester, NY) in November 2006. Bortezomib-resistant SUDHL16-10BR, OCI-LY7-40BR (all GC-DLBCL), and Granta-25BR (MCL) lines were generated as before (21, 29). SUDHL16 (GC-subtype), U2932 (ABC-subtype), and OCI-LY18 (double-hit lymphoma) cells were obtained from the German Collection of Microorganisms (Inhoffenstraße 7B, Germany) in September 2009, March 2013, and August 2013, respectively. SUDHL16-shJNK and SUDHL16-JNK.DN cells were generated as described previously (21). SUDHL4-shHR23B cells were generated by transiently transfecting SUDHL4 cells with shRNA (cat no-KH002801i) construct (SA Biosciences). SUDHL4-shHDAC6 cells were generated by transiently transfecting SUDHL4 cells with shRNA (cat no., TG312491) construct (Origene Technologies). SUDHL4-MEK1 CA cells were generated by stably transfecting SUDHL4 cells as described previously (30). Stable clones were selected by serial dilution using appropriate selection markers (30). All parental cell lines except OCI-LY18 were authenticated by short tandem repeat (STR) DNA fingerprinting using the Promega PowerPlex16HS assay with 15 autosomal loci plus X/Y. All lines were frozen within 2 months of receipt, and fresh aliquots were thawed before lines reached 6 months in culture.

Plasmids and shRNA

Plasmids encoding human HR23B in pCMV6Entry vectors were obtained from Origene Technologies. Four separate sequences were used to knockdown HR23B (i.e., 1, ACGGGTCAGTCTTACGAGAAT; 2, AGTGGTCAATGAACTACATT; 3, CAGCAGATAGGTCGAGAAT; and 4, ACAGTACATCGGGTGATTCTT) and one nonspecific sequence (NC-GGAATCTCATTCGATGCA-T) as negative control. All other constructs were as reported previously (21, 29, 30).

Transient transfections

Transient transfections of SUDHL4 or U2932 cells used a Nucleofector (Lonza). Protocols involved transfection kit C and a cell-specific optimized protocol (O-017) as before (21).

Reagents

Carfilzomib was from Onyx Pharmaceuticals. Ricolinostat was from Acetylon Pharmaceuticals, Inc. 7-Aminoadonycinin D (7-AAD) was purchased from Sigma-Aldrich. All agents were formulated in DMSO. TBAP was from Calbiochem. SB203580 was from Cell Signaling Technology.

Experimental format

Cells were cultured as described earlier (21, 29), and treated with indicated agents and analyzed as below.

Assessment of cell death and apoptosis

Cell viability was monitored by flow cytometry using 7-AAD staining as before (21, 29). Alternatively,
Annexin V/PI staining (both BD PharMingen) was used to monitor apoptosis as before (21). In all studies, 7-AAD and Annexin V/PI assays were concordant.

Collection and processing of primary cells
Studies have been approved by the Investigational Review Board of Virginia Commonwealth University (Richmond, VA). Primary CD34+ cells from healthy volunteers were isolated using an immunomagnetic bead separation technique as described previously (21, 29). Primary DLBCL samples were obtained from ALLcells, LLC.

Separation of S-100 fractions and assessment of cytochrome c release
Cells were harvested and cytosolic S-100 fractions were prepared as before (21). Western blot analysis assessing cytochrome c release was performed as below.

Western blot analysis
Western blot samples were prepared from whole-cell pellets as described previously (21, 29). Source of primary antibodies are presented in Supplementary Methods.

Cell-cycle analysis
Cell-cycle distribution was determined by flow cytometry after fixation and incorporation of propidium iodide (PI) using a commercial software program (Modfit; Becton Dickinson) as per the standard protocol.

Measurement of ROS production
Cells were treated with 20 μmol/L 2,7’-dichlorodihydrofluorescein diacetate for 30 minutes at 37°C. Fluorescence was monitored by flow cytometry and analyzed with CellQuest software (29).

Animal studies
Xenografts were initiated by subcutaneously implanting 5 × 106 Granta-519 cells in 50% Matrigel into the right flank of 10-week-old female severe combined immunodeficient mice (Fox Chase SCID, C.B-17/Icr-Prkdcscid; Charles River) at an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)–certified facility (Charles River Discovery Research Services). In vivo experiments were carried out as described previously (31) and detailed methods are presented in Supplementary Methods.

Proteasome activity assays
Proteasome activity was measured as described previously (21).

Statistical analysis
Synergistic and antagonistic interactions were defined using median dose effect analysis in conjunction with a commercially available software program (CalcuSyn; Biosoft; ref. 32).

Results
Ricolinostat interacts synergistically with carfilzomib in multiple NHL models
Coadministration (48 hours) of minimally toxic concentrations of carfilzomib (e.g., 2.5–3.5 μmol/L) with marginally toxic concentrations of ricolinostat (e.g., 1.5–2.0 μmol/L) substantially increased apoptosis in GC-DLBCL (SUDHL16 and SUDHL4) and ABC-DLBCL cells (U2932; Fig. 1A). Similar results were obtained in MCL cells (Rec-1 and Granta 519) and double-hit DLBCL cells (OCI-LY18; Fig. 1B). Isobologram analysis revealed combination index (CI) values less than 1.0 in SUDHL16 (Fig. 1C) and other cell types (e.g., SUDHL4, U2932; Supplementary Fig. S1), indicating synergistic interactions. Significant increases in ricolinostat lethality in SUDHL16 cells occurred with carfilzomib and ricolinostat concentrations as low as 2.5 μmol/L and 1.5 μmol/L, respectively (Fig. 1D and E), with increased lethality at higher concentrations. Time-course studies of apoptosis induction for the various cell lines are shown in Supplementary Fig. S2A–F. Similar dose–response results were obtained with GC-DLBCL (SUDHL4), ABC-DLBCL (U2932), and double-hit (OCI-LY18) cells (Supplementary Fig. S2A–C). Finally, parallel studies revealed no potentiation of cell death with combined ricolinostat–carfilzomib exposure in normal CD34+ cells, but a sharp increase in primary ABC-DLBCL cells (Fig. 1F).

Similar interactions occur with HDAC6 shRNA knockdown and other HDAC6 or proteasome inhibitors
Parallel studies were performed using SUDHL4 cells in which HDAC6 was knocked down with shRNA (Supplementary Fig. S4A). Compared with scrambled sequence controls, HDAC6 knockdown cells were significantly more sensitive to carfilzomib (2.5 μmol/L)-induced apoptosis (P < 0.04). Similar increases were observed in SUDHL4, SUDHL16, and OCI-LY7 cells coexposed to the HDAC6 inhibitor tubastatin-A and carfilzomib or the proteasome inhibitor MG-132 (Supplementary Fig. S4B). Annexin V/PI staining yielded concordant results in SUDHL4 cells exposed to MG-132 and ricolinostat (Supplementary Fig. S4C), arguing that the observed drug interactions reflect proteasome and HDAC6 inhibition.

Combined ricolinostat–carfilzomib exposure activates stress pathways and increases DNA damage in multiple NHL cell types
Combined carfilzomib–ricolinostat exposure was examined with respect to various cell death/survival pathways in NHL cells. Studies were performed at a treatment interval before the pronounced induction of apoptosis, for example, 24 hours for all cell lines except SUDHL16, in which analysis was performed at 14 hours. Whereas exposure of Granta 519 cells to individual agents had minimal effects, combined treatment markedly increased caspase-3 cleavage and cytochrome c release, consistent with enhanced apoptosis (Fig. 2A). Notably,
cotreatment sharply increased phosphorylation of the stress-related MAPKs JNK (c-Jun N-terminal kinase) and p38 MAPK, accompanied by dephosphorylation of the MAPK extracellular signal–regulating kinase 1/2 (ERK1/2). As anticipated, ricolinostat with or without carfilzomib induced tubulin acetylation. Interestingly, combined exposure induced a pronounced increase in $\gamma$H2A.X expression, indicating double-strand DNA breaks (33).

Virtually identical responses were obtained in GC-DLBCL SUDHL4 cells (Fig. 2B). Similarly, ABC-DLBCL U2932 (Fig. 2C), OCI-LY18 double-hit DLBCL (Fig. 2D), and GC-DLBCL cell SUDHL16 (Supplementary Fig. S5) cells also exhibited sharp increases in JNK and p38 phosphorylation, tubulin acetylation, and $\gamma$H2A.X formation, indicating that the ricolinostat–carfilzomib regimen activates stress pathways and triggers DNA damage in diverse NHL cell types.

To determine whether these signaling perturbations might represent consequences of cell death, SUDHL4, U2932, and SUDHL6 cells were preexposed to the pan-caspase inhibitor BOC-fmk (10 $\mu$mol/L, 3 hours) before treatment. BOC-fmk blocked caspase-3 cleavage, but failed to prevent the activation of the stress kinase JNK, $\gamma$H2A.X upregulation, and dephosphorylation of p-p44/42 (Supplementary Fig. S6) following carfilzomib–ricolinostat exposure. These and the preceding findings argue that the observed signaling events do not simply reflect secondary consequences of apoptosis.

Finally, parallel studies were conducted to assess the functional significance of HDAC6 inhibition on the preceding signaling perturbations. To this end, HDAC6 was knocked down by shRNA in SUDHL4 cells, after which cells were exposed to carfilzomib alone. Compared with scrambled sequence controls, HDAC6 knockdown cells...
treated with carfilzomib alone expressed pronounced JNK and p-38 activation, accompanied by downregulation of p-p44/42 (Supplementary Fig. S7), arguing that the preceding signaling perturbations observed in ricolinostat-treated cells are specifically related to HDAC6 inhibition.

**Carfilzomib–ricolinostat activates stress pathways and triggers DNA damage and cell death in bortezomib-resistant MCL and DLBCL cells**

Parallel studies were performed in previously described bortezomib-resistant DLBCL and MCL cells (21, 29, 31). Whereas ricolinostat or carfilzomib were minimally toxic to SUDHL16-10BR, OCY-LY7-40BR (GC-DLBCL), or Granta-25BR (MCL) cells individually, combined treatment sharply increased cell death (Supplementary Fig. S8A). Like their sensitive counterparts, SUDHL16-10BR cells displayed increases in p-JNK, p-38, and γH2A.X following combined treatment, indicating that bortezomib-resistant NHL cells are sensitive to the ricolinostat–carfilzomib regimen, and that similar mechanisms may be involved (Supplementary Fig. S8B).

**JNK and p38 activation, but not ERK1/2 inactivation, play functional roles in ricolinostat–carfilzomib synergism**

To assess the functional significance of perturbations in MAPks in ricolinostat–carfilzomib lethality toward DLBCL cells, genetic and pharmacologic approaches were used. SUDHL16 cells were stably transfected with JNK1 shRNA plasmids and two clones (C1H1 and C19) were exposed to ricolinostat–carfilzomib, after which cell death

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**Figure 2.** Combined ACY1215/carfilzomib (CFZ) exposure activates stress pathways and increases DNA damage in multiple NHL cell types. A–C, Granta519, SUDHL4, and U2932 cells were treated (24 hours) with carfilzomib (3.5 nmol/L) ± ACY1215 (1.5 μmol/L). D, OCI-LY18 cells were treated (24 hours) with carfilzomib (3.0 nmol/L) ± ACY1215 (2.0 μmol/L). A–D, protein expression was determined by Western blotting using indicated antibodies. Results are representative of three independent experiments. Expression of cytochrome c was studied in cytosolic cell fraction. Expression of total proteins such as JNK P44/42 remain unchanged in U2932 and OCI-LY18 (data not shown); CF, cleaved fragment.
was monitored. JNK1 levels were clearly reduced compared with scrambled sequence controls in transfected cells (Fig. 3A, inset). Notably, both shRNA sublines were significantly less sensitive to combined treatment than controls \( (P < 0.05) \). Parallel studies performed with SUDHL16 cells stably transfected with a dominant-negative JNK construct (C12A and C11B) also displayed significant protection from ricolinostat–carfilzomib lethality compared with controls \( (P < 0.05; \) Fig. 3B). Consistent with these results, JNK shRNA sublines exposed to ricolinostat–carfilzomib displayed diminished caspase-3 cleavage, markedly reduced JNK phosphorylation, and sharply reduced \( \gamma H2A.X \) formation (Fig. 3C). JNK inactivation was also associated with a clear increase in ERK1/2 phosphorylation. Together, these findings argue that JNK activation plays a significant functional role in ricolinostat–carfilzomib lethality in DLBCL cells.

Parallel studies were performed in SUDHL4 cells exposed to ricolinostat–carfilzomib in the presence or absence of the selective p38 inhibitor SB203580. SB203580 clearly diminished p38 phosphorylation and significantly attenuated ricolinostat–carfilzomib lethality \( (P < 0.05; \) Fig. 3D). Consistent with this, SB203580 diminished caspase-3 and PARP cleavage, and also reduced JNK phosphorylation after ricolinostat–carfilzomib exposure (Fig. 3E). In contrast, SUDHL4 cells transiently expressing constitutively active MEK1/2 were equally sensitive to ricolinostat–carfilzomib (Supplementary Fig. S9A) despite the fact that combined treatment with carfilzomib–ricolinostat failed to inhibit enforced expression of p-ERK.

Figure 3. JNK and p38 activation, but not ERK1/2 inactivation, plays functional roles in ACY1215/carfilzomib (CFZ) synergism. A, SUDHL16 cells stably transfected with JNK1 shRNA or scrambled sequence were exposed to carfilzomib (2.5 nmol/L) + ACY1215 (1.5 \( \mu \)mol/L). After 36 hours, cell death was monitored by 7-AAD staining and flow cytometry. Inset, relative expression of JNK1 protein in SUDHL16-scrambled sequence and shJNK clones.

B, SUDHL16 cells stably transfected with JNK-DN cDNA or empty vector (pcDNA3.1) were exposed to carfilzomib (2.5 nmol/L) + ACY1215 (1.5 \( \mu \)mol/L). After 48 hours, cell death was monitored by 7-AAD staining and flow cytometry. Inset, JNK protein expression in SUDHL16 empty vector and JNK-DN clones.

C, following 20 hours of drug exposure as in A above, Western blot analysis was used to monitor expression of the indicated proteins. D, SUDHL4 cells pretreated with the selective p38 inhibitor SB203580 (10 \( \mu \)mol/L) for 2 hours were exposed to carfilzomib (3.0 nmol/L) + ACY1215 (2.0 \( \mu \)mol/L) for 48 hours. After drug exposure, cell death was monitored by 7-AAD staining and flow cytometry. E, following 24 hours of drug exposure as described above (D), Western blot analysis was used to monitor expression of the indicated proteins. All values represent the means of triplicate experiments performed on three separate occasions ± SD. For A and B, * significantly less than values for scrambled or empty vector control lines; \( P < 0.05 \). For D: †, significantly less than values for cells treated with carfilzomib + ACY1215; \( P < 0.05 \).
These findings argue that p38 activation, but not ERK1/2 inactivation, plays a significant functional role in ricolinostat–carfilzomib activity against DLBCL cells, and suggest that p38 acts upstream of JNK.

**Enhanced ROS generation is involved in ricolinostat–carfilzomib lethality**

In view of evidence linking the antitumor activity of both HDAC (34) and proteasome inhibitors (35, 36) to oxidative injury, ricolinostat–carfilzomib effects on ROS generation were examined in DLBCL cells. Carfilzomib–ricolinostat coadministration markedly increased ROS in both SUDHL4 cells and bortezomib-resistant SUDHL16-10BR (Fig. 4A and B), effects largely blocked by the antioxidant TBAP. Notably, TBAP significantly attenuated ricolinostat–carfilzomib lethality in both cell lines ($P < 0.05$; Fig. 4C and D). Moreover, TBAP substantially reduced caspase-3 cleavage, JNK phosphorylation, p-p38 phosphorylation, and γH2A.X formation in ricolinostat–carfilzomib-treated SUDHL4 cells (Fig. 4E). Similar results were obtained in SUDHL16, Granta 519, U2932, and SUDHL16 cells (Supplementary Fig. S10A–C), arguing that oxidative injury plays a significant functional role in ricolinostat–carfilzomib lethality in DLBCL cells.

**Ricolinostat does not increase inhibition of chymotryptic activity by carfilzomib**

To determine whether ricolinostat potentiated proteasome inhibition by carfilzomib, chymotryptic activity assays were performed. Ricolinostat alone had little effect on chymotryptic activity in SUDHL16 or OCI-LY7 cells, whereas carfilzomib markedly reduced activity in both lines (4 hours) treatment (Supplementary Table S1).

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Figure 4. Simultaneous carfilzomib (CFZ) and ACY1215 exposure induces oxidative injury-mediated cell death in both parental and bortezomib-resistant cells. SUDHL4 (A) and SUDHL16-10BR (B) cells were treated with carfilzomib (3.5 nmol/L) + ACY1215 (2.0 μmol/L) and carfilzomib (5 nmol/L) + ACY1215 (1.5 μmol/L), respectively (±pretreatment with 400 μmol/L TBAP for 3 hours) for 12 hours, after which ROS generation was monitored. SUDHL4 (C) and SUDHL16-10BR (D) cells were treated with carfilzomib (3.0 nmol/L) ± ACY1215 (2.0 μmol/L) and carfilzomib (5 nmol/L) ± ACY1215 (1.5 μmol/L), respectively (±pretreatment with 400 μmol/L TBAP for 3 hours) for 48 hours, after which cell death was monitored by 7-AAD. Values represent mean ± SD for triplicate determinations from three independent experiments. E, following 24 hours of drug exposure as in A, expression of the indicated proteins was monitored by Western blotting. For C and D: **, significantly more versus carfilzomib or ACY1215 single-agent treatment; $P < 0.01$; *, significantly less in the presence of TBAP versus carfilzomib + ACY1215 treatment alone; $P < 0.05$. 2892 Mol Cancer Ther; 13(12) December 2014 Molecular Cancer Therapeutics
However, combined exposure did not reduce chymotryptic activity further ($P > 0.05$). Longer drug exposure intervals beyond 4 hours did not induce any further reduction in chymotryptic activity (data not shown). This argues that the enhanced lethality of the combination does not stem from increased chymotryptic inhibition.

**Combined ricolinostat–carfilzomib exposure leads to G2–M arrest of DLBCL cells**

Cell-cycle analysis revealed that ricolinostat alone had modest effects on cell-cycle progression of SUDHL4 and OCI-LY7 cells leading to an increase in the G0–G1 population with a clear decrease in S-phase cells, whereas carfilzomib induced modest increases in the G2–M population (Supplementary Table S2). However, combined exposure dramatically increased G2–M cells, while reducing the G0–G1 and S-phase populations (Supplementary Table S2), raising the possibility that increased ROS generation and marked DNA damage induced by this regimen may trigger G2–M arrest. In support of this notion, pretreatment of cells with the antioxidant TBAP circumvented G2–M arrest of the cells following exposure to the carfilzomib–ricolinostat regimen (Supplementary Table S2) and also prevent induction of γH2A.X (Fig. 4E).

**Induction of DNA damage plays a functional role in ricolinostat and carfilzomib lethality**

To assess the functional role of DNA damage in carfilzomib–ricolinostat lethality, the DNA damage linker Histone 1.2 was knocked down in U2932 cells. Knockdown of Histone 1.2 significantly reduced carfilzomib–ricolinostat lethality (Supplementary Fig. S11A), and diminished PARP and caspase-3 cleavage (Supplementary Fig. S11B), supporting a functional role for DNA damage in cell death induction by this regimen.

**Combined ricolinostat–carfilzomib exposure downregulates the cargo protein HR23B, which contributes to cell death**

Previous studies have highlighted a role for the cargo protein HR23B in the sensitivity of cutaneous T-cell lymphoma cells to pan-HDACIs (37, 38). To determine whether this protein might play a role in ricolinostat–carfilzomib lethality in DLBCL cells, Western blot analysis of HR23B was performed. Exposure of SUDHL16, SUDHL4, and OCI-LY7 cells to ricolinostat and carfilzomib in combination, but not individually, markedly reduced HR23B expression (Fig. 5A). Of note, shRNA-mediated HR23B knockdown in SUDHL4 cells significantly increased cell death compared with carfilzomib or ricolinostat alone, NC shHR23B

**Figure 5.** Combined ACY1215/carfilzomib exposure downregulates the cargo protein HR23B, contributing to cell death. A, cells were treated with carfilzomib (SUDHL16, 2.5 nmol/L; SUDHL4 and OCI-LY7, 3.5 nmol/L) in the presence or absence of ACY1215 (1.5–2.0 μmol/L) for 14 hours (SUDHL16 cells) to 24 hours (SUDHL4 and OCI-LY7 cells). Protein expression was determined by Western blotting. Each lane was loaded with 20 μg of protein; blots were stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer. Results are representative of three independent experiments. B, SUDHL4 cells were transiently transfected with shHR23B or scrambled control sequence for 24 hours, and then exposed to drug concentrations as in A for an additional 48 hours. Cell death was determined by flow cytometry with 7-AAD staining. * significantly more for shHR23B sublines compared with scrambled controls; $P < 0.05$. C, SUDHL4 cells were transfected and treated as described in B above for 24 hours and expression of the indicated proteins was monitored by Western blotting.
and with the ricolinostat–carfilzomib combination ($P < 0.05$ in each case; Fig. 5B). This was associated with increased caspase-3 cleavage and p-JNK levels (Fig. 5C), raising the possibility that downregulation of the cargo-loading protein HR23B may contribute to ricolinostat–carfilzomib lethality in DLBCL cells.

**Ricolinostat enhances the in vivo activity of carfilzomib in a MCL model**

To assess the *in vivo* activity of the ricolinostat–carfilzomib regimen, a MCL xenograft flank model was used as previously described (29). Individual treatment with ricolinostat (50 mg/kg) or carfilzomib (1.0 mg/kg) minimally reduced tumor volumes compared with controls (21.2% and 18.5% tumor growth inhibition versus vehicle alone, respectively; Fig. 6A). However, combined treatment resulted in a significant further reduction in tumor volumes compared with individual treatment as assessed by two-way ANOVA with the Tukey multiple comparison correction ($P < 0.001$ at day 21 for the combination vs. single agents; 42% tumor growth inhibition vs. vehicle alone). Moreover, animal survival with both agents was significantly greater than that observed with either single agent (log-rank test; $P < 0.05$; Fig. 6B). Western blot analysis performed on tumor lysates revealed modest but discernible increases in expression of cell death marker such as PARP cleavage, caspase-3 activation, induction of p-JNK and γH2A.X in tumors obtained from animals treated with both agents (Fig. 6C). Finally, animal body weight loss was minimal with single-agent or combination treatment (e.g., <10%) and quickly recovered (Fig. 6D). Together, these findings indicate that combination treatment with ricolinostat and carfilzomib has potential therapeutic efficacy in a MCL xenograft model.
that the ricolinostat–carfilzomib regimen is tolerable in animals and results in enhanced antitumor effects compared with treatment with either individual agent similar to what has been observed in vitro.

Discussion

The present studies indicate that the novel HDACI ricolinostat, which preferentially inhibits class IIb HDAC6 and acts as a tubulin acetylase (28), interacts synergistically with the irreversible proteasome inhibitor carfilzomib to induce cell death in diverse NHL cells, including bortezomib-resistant cells. These studies were prompted by several considerations. First, more selective HDACIs may exhibit less toxicity than pan-HDACIs. Second, irreversible proteasome inhibitors may overcome resistance to other agents in this class. Numerous studies have documented synergistic interactions between pan-HDACIs and proteasome inhibitors in malignant hematopoietic cells (21, 25, 35), and we have previously described synergism between carfilzomib and vorinostat in both DLBCL and MCL models in vitro and in vivo (21, 29). Moreover, synergistic interactions between ricolinostat and bortezomib in multiple myeloma cells have recently been described (28). Mechanisms underlying such interactions are likely multifactorial, for example, oxidative injury (25, 29, 35), inhibition of NF-κB (21, 29), and potentially downregulation of DNMT1 (39) by proteasome inhibitors, which may cooperate with HDAC inhibition to promote expression of cell death and differentiation-related genes (40). In addition, HDACIs which target HDAC6 may disrupt the dynein motor and induce aggresome dysfunction by affecting cellular protein disposition (41). This effect may amplify the lethal consequences of proteasome inhibition by interfering with alternative mechanisms for misfolded protein elimination (i.e., the aggresome), culminating in proteotoxic stress (42). Indeed, activation of multiple stress-related pathways by the ricolinostat–carfilzomib regimen is consistent with the latter mechanism. The present studies, including the observation that HDAC6 knockdown or an HDAC6-selective inhibitor (i.e., tubastatin-A) sensitized cells to carfilzomib support the notion that HDAC6 inhibition plays a significant functional role in HDACI–carfilzomib interactions.

It is noteworthy that this strategy was effective across a range of NHL models, including DLBCL cells and MCL cells. Significantly, the regimen appeared equally active against ABC-DLBCL cells and GC-DLBCL cells. The ABC-DLBCL subtype is associated with NF-κB dependence, and generally inferior responses to chemotherapy associated with poor prognosis (1, 2). In addition, ricolinostat–carfilzomib was active against models of double-hit DLBCL, which is characterized by lack of response to standard chemotherapy and a particularly grim prognosis (3). Finally, the regimen robustly induced cell death in bortezomib-resistant DLBCL and MCL lymphoma cells, accompanied by pharmacodynamic events similar to those observed in their bortezomib-sensitive counterparts. Previous studies in multiple myeloma cells indicate that carfilzomib can at least partially circumvent resistance or unresponsiveness to bortezomib (11, 43), raising the possibility that this capacity may contribute to the activity of the ricolinostat–carfilzomib regimen in resistant DLBCL or MCL cells.

The ricolinostat–carfilzomib regimen activated multiple stress-related pathways associated with oxidative injury and DNA damage, and these events appeared to play significant functional roles in cell death. Specifically, activation of the JNK1/2 pathway in response to diverse stresses is an important mediator of cell death (44), and the relative activities of the JNK1/2 and ERK1/2 pathways have been postulated to determine cell fate (45). Analogously, p38 is activated in response to diverse cellular stresses, and is generally associated with cell death (45). Notably, genetic or pharmacologic interruption of these pathways significantly attenuated ricolinostat–carfilzomib lethality, arguing for functional roles for these stress kinases in the pathways leading to cell death. In contrast, enforced activation of ERK1/2, which generally inhibits apoptosis (46), failed to diminish ricolinostat–carfilzomib lethality, suggesting that ERK1/2 inactivation represents a secondary event. It is noteworthy that combined ricolinostat–carfilzomib triggered marked oxidative injury (e.g., ROS generation), which played an important functional role in cell death. In this context, both proteasome (7) and HDAC inhibitor (47) lethality has previously been linked to oxidative injury and induction of DNA damage (e.g., γH2AX formation; ref. 33). Significantly, antioxidants blocked not only ROS generation and cell death, but also stress pathway activation (e.g., JNK1/2 phosphorylation) and induction of DNA damage (e.g., γH2AX formation), suggesting a hierarchical relationship in which oxidative injury plays an apical role while activation of stress pathways transmits this signal to the cell death machinery. The observation that Histone1.2 shRNA knockdown partially but significantly diminished carfilzomib–ricolinostat lethality suggests a functional role for DNA damage (i.e., γH2AX formation) in synergistic interactions between carfilzomib and ricolinostat. On the other hand, as JNK knockdown also circumvented γH2AX induction, it is plausible that the latter is influenced by both DNA damage and JNK-dependent phosphorylation.

As both HDAC6 and proteasome inhibitors are both known regulators of endoplasmic reticulum (ER) stress (7, 26), it is possible that this process might have contributed to lethality. However, while single-agent carfilzomib or ricolinostat treatment induced modest increases in protein accumulation and also slightly increased CHOP expression, minimal further increases were observed with combined treatment (data not shown). This argues, albeit indirectly, against a predominant role for ER stress–related events as a basis for synergism. However, a role for ER stress or aggresome dysfunction in the lethal effects of this regimen cannot presently be excluded.
Several lines of evidence suggest that the ricolinostat–carfilzomib regimen preferentially targets NHL cells. Previous studies suggest that both proteasome (8) and pan-HDAC (48) inhibitors selectively induce transformed cell death. Notably, the ricolinostat–carfilzomib regimen was active against a broad array of NHL cell models, as well as primary DLBCL cells, but minimally toxic to normal hematopoietic cells. In addition, ricolinostat administered with or without carfilzomib exhibited minimal toxicity in animals. However, combined exposure induced more pronounced tumor growth suppression in a xenograft MCL model and a significant improvement in survival compared with single agents, arguing that this strategy may preferentially kill lymphoma versus normal cells. In this context, a recent phase I trial in patients with refractory NHL has demonstrated that a regimen combining carfilzomib with the pan-HDACI vorinostat is tolerable and displays some, albeit modest, activity (49). If a more selective HDACI induces less toxicity, higher drug doses and greater regimen activity may be possible. Finally, several pharmacodynamic markers of regimen activity identified in vitro studies (e.g., enhanced JNK1/2 phosphorylation and increased γH2AX.X formation) were observed in tumor specimens obtained from animals treated with agents in vivo. Such pharmacodynamic events could serve as biomarkers in future trials. Collectively, these findings suggest that a strategy combining the HDAC6 inhibitor ricolinostat with carfilzomib warrants consideration for the treatment of refractory NHL, and plans to explore this possibility are currently underway.

Disclosure of Potential Conflicts of Interest
S.N. Quayle and S.S. Jones have ownership interest (including patents) in Acetylgon Pharmaceuticals Inc. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Dasmahapatra, S.N. Quayle
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References
Carfilzomib and Ricolinostat in NHL Cells


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