Dual Targeting of Protein Degradation Pathways with the Selective HDAC6 Inhibitor ACY-1215 and Bortezomib Is Synergistic in Lymphoma

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Abstract

Purpose: Pan-class histone deacetylase (HDAC) inhibitors are effective treatments for select lymphomas. Isoform-selective HDAC inhibitors are emerging as potentially more targeted agents. HDAC6 is a class IIb deacetylase that facilitates misfolded protein transport to the aggresome for degradation. We investigated the mechanism and therapeutic impact of the selective HDAC6 inhibitor ACY-1215 alone and in combination with bortezomib in preclinical models of lymphoma.

Experimental Design: Concentration–effect relationships were defined for ACY-1215 across 16 lymphoma cell lines and for synergy with bortezomib. Mechanism was interrogated by immunoblot and flow cytometry. An in vivo xenograft model of DLBCL was used to confirm in vitro findings. A collection of primary lymphoma samples were surveyed for markers of the unfolded protein response (UPR).

Results: Concentration–effect relationships defined maximal cytotoxicity at 48 hours with IC₅₀ values ranging from 0.9 to 4.7 μmol/L. Strong synergy was observed in combination with bortezomib. Treatment with ACY-1215 led to inhibition of the aggresome evidenced by acetylated α-tubulin and accumulated polyubiquitinated proteins and upregulation of the UPR. All pharmacodynamic effects were enhanced with the addition of bortezomib. Findings were validated in vivo where mice treated with the combination demonstrated significant tumor growth delay and prolonged overall survival. Evaluation of a collection of primary lymphoma samples for markers of the UPR revealed increased HDAC6, GRP78, and XBP-1 expression as compared with reactive lymphoid tissue.

Conclusions: These data are the first results to demonstrate that dual targeting of protein degradation pathways represents an innovative and rational approach for the treatment of lymphoma.

Introduction

Targeting protein degradation pathways has proven to be an effective therapeutic strategy for many types of hematologic malignancies (1–4). As our understanding of this complex biology deepens, many novel targets have emerged which may complement other validated approaches, like targeting the 26S proteasome. HDAC6 is a class IIb deacetylase that facilitates misfolded protein transport to the aggresome for proteasome-independent proteolysis. Its inhibition activates the unfolded protein response (UPR), a cellular quality control mechanism with 2 primary functions: (i) to promote survival during endoplasmic reticulum (ER) stress by chaperoning proteins for refolding and halting transcription and translation until homeostasis is restored and (ii) to signal CHOP (C/EBP homologous protein)-mediated apoptosis when homeostasis cannot be reestablished (5–9).

The development of pan-class I/II deacetylase (HDAC) inhibitors like vorinostat, romidespin, and belinostat have led to FDA indications in T-cell–derived malignancies (10–13). Given these drugs have highly pleiotropic effects, indiscriminate inhibition of specific deacetylases could lead to off-target effects and potential untoward toxicity. The development of isoform-selective HDAC inhibitors has created the opportunity to dissect the specific functions of the many HDAC isoforms, allowing for more precise targeting of discrete cellular operations with potentially less toxicity (14–16). It is now well recognized that HDAC inhibitors not only exhibit epigenetic effects but also catalyze important posttranslational modifications that can affect the function of transcription factors (p53), oncogenes (Bcl6), and protein degradation pathways (aggresome and UPR; refs. 17–19).

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Translational Relevance

Given the high rate of protein and immunoglobulin synthesis in lymphocytes and by extension lymphoid neoplasia, the lymphomas can be characterized by a dependency on protein degradation systems to manage the constant load of potentially toxic misfolded proteins. Basal expression of markers of the unfolded protein response (UPR) pathway is upregulated in primary patient lymphoma samples as compared with reactive lymph nodes. Targeting the UPR with the selective HDAC6 inhibitor ACY-1215 leads to apoptosis in models of lymphoma. Dual targeting of protein degradation systems with the proteasome inhibitor bortezomib and ACY-1215 leads to synergistic antitumor activity in both in vitro and in vivo assays. Mechanistically, apoptosis is mediated through release of PERK from GRP78 and activation of the PERK–proapoptotic pathway. ACY-1215 is currently being studied in a phase I/II clinical trial in patients with relapsed and refractory lymphoma.

While protein turnover occurs primarily through the ubiquitin–proteasome pathway, under stress conditions where the proteasome is physiologically overwhelmed or therapeutically inhibited, the aggresome pathway is recruited to manage intracellular protein dynamics (20, 21). While most cells depend on the UPR to coordinate proteostasis, lymphocytes physiologically upregulate the UPR–homeostasis pathway and downregulate UPR-induced apoptosis to allow for generation of high-affinity antibodies. Like somatic hypermutation, this natural feature of lymphocyte biology can have an oncogenic consequence. The hypothesis that emerges is that if lymphoid malignancies can be characterized by the production of high levels of proteins reminiscent of natural lymphocyte function, then complete pharmacologic inhibition of proteolytic operations would lead to an induction of the UPR and cell death. We present the first data to establish the importance of this biology, and its targeting, in lymphoid malignancies.

ACY-1215 is a specific HDAC6 inhibitor that was initially evaluated in multiple myeloma (22, 23). In preclinical models of lymphoma, we have demonstrated that selectively targeting HDAC6 with ACY-1215 inhibits sequestration of misfolded proteins by disrupting transport to the aggresome through acetylation of α-tubulin. ACY-1215 demonstrated activity in diffuse large B-cell lymphoma (DLBCL), both germinal center B-cell (GC) DLBCL cell lines; Hbl-1, Hbl-2, Jeko-1, Jvm-2, and Rec-1 are GC cell lines; CCL-119, H9, HH, and Sup-T1 are T-cell lymphoma (TCL) cell lines. Hbl-1, H9, HH, Sup-T1, and Sup-T1 were obtained from ATCC. OCI-Ly1, OCI-Ly7, and OCI-Ly10 were obtained from DSMZ. All cell lines were authenticated by a board-certified hematopathologist including verification of morphology and immunophenotype annually. CCL-119, Hbl-1, Hbl-2, H9, HH, Jeko-1, Jvm-2, Mav-1, Rec-1, Sup-DHL2, Sup-DHL4, Sup-DHL6, and Sup-T1 were grown in RPMI (10% FBS). OCI-Ly1, OCI-Ly7, OCI-Ly10, and Riva were grown in IMDM (10% FBS).

Cell viability assays

Cells (3 × 10^5 cells per well) were incubated with 1:100 dilution of ACY-1215, bortezomib, or both. Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation) and confirmed by Vi-Cell Series Cell Viability Analyzer (24, 25).

Flow cytometry

FACS Calibur System was used to acquire the fluorescence signals (≥1 × 10^5 events/sample) and data were analyzed using FlowJo 8.8.6. Cells (3 × 10^5/mL) were quantitated for apoptosis using Alex Fluor 488/Annexin V (Dead cell apoptosis kit; Invitrogen #V13240).

Western blotting and immunoprecipitation

Western blotting was performed as previously described (26). Antibodies obtained: anti-acetylated lysine, acetyl-histone 3 (Lys9), anti-caspase-3, anti-PARP, anti-Bcl-2, anti-BIM, anti-GRP78/BIP, anti-HDAC6, anti-CHOP/GADD153, anti-PERK, anti-p-eif2α, anti-p-eIRe, anti-XBP-1, anti-ubiquitin, anti-β-actin (Cell Signaling Technology); anti-acetyl-α-tubulin and anti-α-tubulin (Sigma–Aldrich); and Protein G-Agarose Beads (Santa Cruz). For detection of acetylated-GRP78 and the GRP78:PERK protein complex immunoprecipitation (IP) was performed as previously described on 4 × 10^5 OCI-Ly10 cells (9). Densitometry analysis was performed on scanned images using the ImageJ software (NIH; ref. 27).

In vivo studies

Animals were housed and maintained in accordance with and under an IACUC-approved protocol. A total of 1 × 10^7 OCI-Ly10 (50% Matrigel; BD Biosciences) were subcutaneously injected into the flanks of 5- to 7-week-old beige/SCID mice (Taconic Farms, Inc.). Treatment was initiated when tumor volume measured 200 mm^3. Tumors volume was assessed using the 2 largest perpendicular axes (l = length; w = width) and calculated using the formula v = 0.5 l^2 w. Mice were divided into 4 cohorts of 8 to 10 mice per cohort as follows: (i) control: normal saline days 1–5, 8–12, 15–19; (ii) ACY-1215 (A): 50 mg/kg days 1–5, 8–12, 15–19; (iii) bortezomib (B): 0.5 mg/kg/day 1, 8, 11; and (iv) A + B. Drugs were

Materials and Methods

Drugs and reagents

ACY-1215 was provided by Acetylon Pharmaceuticals, Inc. Bortezomib (Velcade) was obtained from Selleck Chemicals. Both drugs were diluted in DMSO.

Cell lines and culture

Hbl-1, OCI-Ly10, Riva, and Su-DHIL2 are ABC DLBCL cell lines; OCI-Ly1, OCI-Ly7, Su-DHIL4, and Su-DHIL6 are germinal center (GC) DLBCL cell lines; Hbl-2, Jeko-1, Jvm-2, and Rec-1 are MCL cell lines; CCL-119, H9, HH, and Sup-T1 are T-cell lymphoma (TCL) cell lines. Hbl-1, Riva, Su-DHIL-2, Su-DHIL-4, Su-DHIL-6, Hbl2, Jeko-1, JVM-2, Rec-1, CCL-119, H9, HH, and Sup-T1 were obtained from ATCC. OCI-Ly1, OCI-Ly7, and OCI-Ly10 were obtained from DSMZ. All cell lines were authenticated by a board-certified hematopathologist including verification of morphology and immunophenotype annually. CCL-119, Hbl-1, Hbl-2, H9, HH, Jeko-1, Jvm-2, Mav-1, Rec-1, Su-DHIL2, Su-DHIL4, Su-DHIL6, and Sup-T1 were grown in RPMI (10% FBS). OCI-Ly1, OCI-Ly7, OCI-Ly10, and Riva were grown in IMDM (10% FBS).

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diluted in normal saline 0.9% and were administered via the intraperitoneal route. Mice were assessed for weight loss and tumor volume 3 times a week. Animals were sacrificed when the tumor volume exceeded 2,000 mm³ or after sustained loss of >10% body weight in accordance of institutional guidelines.

Pharmacokinetic/Pharmacodynamic in vivo studies
Mice were studied for pharmacokinetic and pharmacodynamic effects of ACY-1215. Approximately 250 µL of blood was collected by jugular vein bleed at 0.5, 1, and 2 hours after treatment. Mice were sacrificed at 4, 6, and 8 hours after treatment; blood, tumor tissue, and spleens were collected for measurement of drug concentration, immunohistochemistry (IHC), and Western blot analysis.

LC-MS/MS method for simultaneous determination of ACY-1215 and bortezomib in serum and tissue
Bortezomib and ACY-1215 were extracted as described previously with modifications (16, 28). Four volumes of acetonitrile:methanol:formic acid (50/50/0.1 v/v/v) were added to 250 µL of serum or 500 µL of aqueous tissue homogenate containing 100-mg wet tissue disrupted by polytron homogenizer. Experiments were carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters). The system was controlled by Mass Lynx Software version 4. 1. Samples were maintained at 4°C and 5 µL was loaded onto a Waters ACQUITY UPLC BEH Phenyl column (3 mm inner diameter × 100 mm with 1.7-µm particles). Positive electrospray ionization (ESI+) with multiple reaction monitoring (MRM) mode was performed using the parameters: capillary voltage, 4.0 kV; source temperature, 150°C; desolvation temperature, 500°C; desolvation gas flow, 1,000 L/h; and collision gas flow, 0.15 mL/min. Correction for possible matrix effect was performed.

Primary lymphoma samples
Representative primary human lymphoma samples were obtained from the Department of Pathology and Cell Biology archives, Columbia University Medical Center (New York), under an Institutional Review Board–approved protocol and verified by IHC.

IHC staining
All tissue samples (mouse and human) were processed as 5-mm-thick FFPE tissue sections and stained by the pathology core laboratory. Antibodies included acetylated α-tubulin (Sigma 17451) 1:1,000 for 90 minutes at 4°C, GRP78 (Santa Cruz sc-1050) 1:250 overnight at 4°C, CytC (Cell Signaling) 1:100 90 minutes at room temperature, and CHOP (Cell Signaling) 1:300 90 minutes at room temperature). After washing, biotinylated secondary antibody (Vector Laboratories) and diaminobenzidine (Dako) were applied and then counterstained with Harris hematoxylin. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed by the pathology core facility in accordance with the manufacturer’s instructions (Roche 11068408170910).

All samples were evaluated and marker analysis results were recorded in a double blind fashion by 2 independent investigators at 2 different time points. A positive result was defined as more than 20% tumor cells demonstrating protein expression (29, 30). Samples were scored in a semiquantitative manner into high intensity staining (+2) or low intensity staining (+1). Staining results were correlated with clinical data. Images were obtained with an Olympus BX41 microscope and captured with Camera Olympus q capture, q color-3 at 20× and 40× magnification.

Statistical analysis
For determination of the inhibitory concentration of 50% of cells (IC50) and synergy, all experiments were run in triplicate and repeated at least twice. IC50 was calculated with CalcuSyn software (Biosoft). Synergy was computed using the Excess over Bliss (EOB) method. Bliss independence was determined using the formula C = A + B – A × B; where C designates the combined response for the 2 single compounds with effects A and B. An EOB >10 connotes synergy (31–33). Flow cytometric assays were performed in doublet, repeated at least twice, and reported as the mean with associated SDs.

Results
ACY-1215 is a selective HDAC6 inhibitor with anti-lymphoma activity
Selective activity of ACY-1215 was determined in 4 distinct lymphoma subtypes (ABC-DLCL: OCI-Ly10, GC-DLCL: OCI-Ly7, MCL: HBL-2, TCL: H9) by culturing cells with increasing concentrations of ACY-1215 for 24 hours (Fig. 1A). This resulted in a concentration-dependent accumulation of acetylated α-tubulin, a specific substrate of HDAC6, in all cell lines. OCI-Ly10 and OCI-Ly7 cells were cultured with ACY-1215 or romidepsin for 24 hours (Fig. 1A). After treatment with ACY-1215 (isoform-selective HDAC inhibitor), there was minimal acetylation of histone-3 (H3), whereas both lines exhibited a substantial increase in acetylated-H3 following treatment with the pan-class I/II HDAC inhibitor romidepsin. In contrast, romidepsin induced minimal acetylation of α-tubulin. These data establish that ACY-1215, while bearing structural similarity to several pan-class HDAC inhibitors, selectively targets HDAC6 and does not exhibit the same pharmacologic effects seen with other pan-class HDAC inhibitors.

The concentration–effect relationship of ACY-1215 on lymphoma cell viability was determined at 24, 48, and 72 hours after treatment for 8 DLBCL cell lines (GC:OCI-Ly1, OCI-Ly7, Su-DHL4 and Su-DHL6; ABC: Hbl-1, OCI-Ly10, RIVA, and Su-DHL2), 4 MCL cell lines (Hbl-2, Jeko-1, Jym-2, and Rec-1), and 4 TCL cell lines (CCL-119, H9, HH, and Sup-T1) totaling 16 cell lines across 4 distinct subtypes of lymphoma. ACY-1215 demonstrated a concentration- and time-dependent effect, whereas there was no effect on peripheral blood mononuclear cells (PBMC; Supplementary Fig. S1). The maximal cytotoxic response was induced in all cell lines by 48 to 72 hours, with IC50 values...
Selective HDAC6 inhibition with ACY-1215 has activity in lymphoma. A, concentration–effect relationship of treatment with increasing concentrations of ACY-1215 on acetylation of α-tubulin after 24 hours in 4 lymphoma cell lines (OCI-Ly10, OCI-Ly7, HBL-2, H9). ACY-1215 selectively targets α-tubulin and not histone 3 as compared with romidepsin after 24-hour exposure in OCI-Ly10 and OCI-Ly7. B, ACY-1215 has single-agent cytotoxic effect across a large panel of lymphoma cell lines. Luminometric assay growth inhibition curves of ACY-1215 on 16 lymphoma cell lines at 48 hours. Growth inhibition IC50 mean values (μmol/L) in 16 cell lines at 48 hours is reported in parenthesis. C, PARP and caspase-3, -8, and -9 cleavage was demonstrated for cells treated with ACY-1215 at 48 hours as measured by Western blot analysis. D, basal levels GRP78 expression is higher across 8 cell lines as compared with PBMCs. Basal HDAC6 level does not correlate with response to treatment with ACY-1215. Densitometry analysis was performed on scanned immunoblot images using the ImageJ software (NIH). Relative levels of protein expression were calculated and plotted.
ranging from 0.9 to 4.7 μmol/L after 48-hour exposure and from 0.9 to 5 μmol/L after a 72-hour exposure. Interestingly, there was limited variability in IC50 to ACY-1215 across the panel of cell lines, with the ABC-DLBCL line OCI-Ly10 being the most sensitive and the GC-DLBCL line Su-DHL4 being relatively more resistant (Fig. 1B). An increase in caspase-3, caspase-8, caspase-9, and PARP cleavage was observed in all cell lines (OCI-Ly10, OCI-Ly7, HBL2, H9) treated with ACY-1215 at increasing concentrations after 48-hour exposure (Fig. 1C).

Eight cell lines were compared with PBMC extracts for expression of GRP78, a protein considered to be the master regulator of the UPR. Increased expression of GRP78 was observed across all lymphoma cell lines compared with PBMCs (Fig. 1D). Interestingly, there was variable expression of HDAC6 across cell lines and the level of HDAC6 expression did not correlate with sensitivity to ACY-1215.

Dual targeting of protein degradation pathways with ACY-1215 and bortezomib is synergistic in vitro

Four lymphoma cell lines (OCI-Ly10, OCI-Ly7, HBL-2, H9) treated with ACY-1215 ±bortezomib at increasing concentrations and analyzed at 24, 48, and 72 hours. Strong synergy was demonstrated in all 4 cell lines at 48 hours (Fig. 2A). EOB reached as high as 41 (strong synergy) for OCI-Ly7 (DLBCL) following 48-hour exposure to 1,250 nmol/L ACY-1215 and 4 nmol/L bortezomib (EOB values > 10 = synergy). Similar

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Dual targeting of protein degradation pathways with ACY-1215 and bortezomib is synergistic. A, four lymphoma cell lines representing ABC-DLBCL, GC-DLBCL, MCL, and TCL (OCI-Ly10, OCI-Ly7, HBL-2, H9, respectively) were treated with increasing concentrations of bortezomib 0 to 5 nmol/L and ACY-1215 0 to 1,500 nmol/L. Cell viability was measured by luminometric assays at 48 hours. Values represent means expressed as percentages compared with the untreated control; error bars represent SD. Synergy was calculated by EOB independence where values greater than 10 represent synergy. Synergy was achieved across all cell lines with EOB as high as 41 in OCI-Ly7. B, assessment of apoptosis by Annexin V and propidium iodine in 4 cell lines mentioned in A. Cells were incubated with ACY-1215 (1 or 1.5 μmol/L) or bortezomib (2–5 nmol/L) at their approximate IC10–20 or the combination. Compared with the untreated control, the combination resulted in increased apoptosis across all cell lines at 48 hours. C, PARP and caspase-3 cleavage was demonstrated for cells treated with the combination of ACY-1215 and bortezomib at 48 hours across cell lines.
Figure 3.
Targeting with ACY-1215 and bortezomib leads to marked activation of the UPR. A, treatment of OCI-Ly10 cells with 2 μmol/L ACY-1215 for 6 and 24 hours. At 6 hours, acetylation of GRP78 was demonstrated by immunoprecipitation with GRP and immunoblot with acetyl-lysine. (Continued on the following page.)
results were observed across cell lines with EOB of 25 and 20 in ABC-DLBCL and TCL, respectively, but no effect or evidence of synergy was observed in PBMCs (Supplementary Fig. S1). Schedule of administration was evaluated systematically with simultaneous exposure leading to the greatest synergy (data not shown). Cell death occurred by apoptosis across all cell lines studied. As an example, OCI-Ly10 cultured with ACY-1215, bortezomib, or the combination led to induction of apoptosis in 9%, 20%, and 67%, respectively (Fig. 2B). The combination also induced marked caspase-3 and PARP cleavage (Fig. 2C).

Dual targeting of protein degradation pathways with ACY-1215 plus bortezomib leads to marked activation of the UPR

Treatment with ACY-1215 acts directly on the master regulator of the UPR, GRP78. There was evidence of 190% increase in acetylation of GRP78 after 6 hours of exposure as compared with control, with subsequent dissociation of PERK at 24 hours by 54% (Continued. By 24 hours, PERK dissociated from GRP78 as measured by co-immunoprecipitation. Densitometry analysis was performed on scanned immunoblot images using the ImageJ software (NIH). Relative levels of protein expression were calculated and plotted. Treatment of OCI-Ly10 and OCI-Ly7 with 2 μmol/L ACY-1215 for 24 hours led to upregulation of the UPR demonstrated by increased expression of GRP78, PERK, p-eIF2α, and CHOP as measured by immunoblot. B, treatment with the combination of 1.0 μmol/L ACY-1215 and 2 nmol/L bortezomib led to increased accumulation of polyubiquinated protein compared with treatment with either drug alone in OCI-Ly10 and OCI-Ly7 cell lines at 48 hours. C, the combination of ACY-1215 and bortezomib led to synergistic induction of the UPR demonstrated by increased expression of GRP78, PERK, p-eIF2α, p-IRE1, and spliced XBP-1 as measured by immunoblot at 36 hours. D, schema of hypothesized mechanism of action of ACY-1215 on modulating the aggresome and the UPR. Treatment with ACY-1215 leads to inhibition of HDAC6 leading to acetylation of α-tubulin and GRP78. Acetylation of α-tubulin leads to disruption of the tubulin–HDAC6–motor dynein complex preventing misfolded proteins from aggresome-mediated degradation. Accumulated misfolded proteins then activate the UPR, a quality control mechanism in cells to shuttle proteins back to the endoplasmic reticulum for refolding, or to trigger apoptosis if proteostasis cannot be maintained. ACY-1215 leads to acetylation of GRP78, which releases key regulators of the UPR: IRE1, ATF6, and PERK.

Figure 4.

ACY-1215 plus bortezomib led to significant tumor growth delay and prolonged OS in a xenograft model of DLBCL (OCI-Ly10). A, weight was recorded every 72 hours. All treatment groups had decreased weight as compared with untreated mice. Mice that had more than 10% weight loss within the first week regained their weight on day 10. There was only 1 toxic death among all mice treated with ACY-1215 in both the single-agent and combination cohorts (n = 19). B, after 1 cycle of therapy, the combination treatment with ACY-1215 and bortezomib led to a significant tumor growth delay compared with either drug alone and untreated control mice (P = 0.006). Mean doubling time was significantly prolonged in the combination after 1 cycle of treatment (16.71 days) compared with ACY-1215 (10.68) and bortezomib (10.42) or control (9.4). C, OS was prolonged after 1 cycle with the combination with median OS of 42 days compared with 29.5, 31, and 33 days for control, ACY-1215, and bortezomib cohorts (P < 0.05).
Figure 5.
Pharmacokinetic and pharmacodynamic effects of ACY-1215 in mice. A, serum and tumor tissue were collected from mice at sequential time points and analyzed for concentration of ACY-1215 and bortezomib by LC-MS/MS. Mice were treated with ACY-1215 at 0.5 or 50 mg/kg alone and 50 mg/kg ACY-1215 with 0.5 mg/kg bortezomib. Drug concentrations are represented as mean values with SD where more than one mouse sample was available. Cmax in serum was determined at 0.5 hours after injection at 10,888 nmol/L. B, graphical representation of ACY-1215 concentration over time. Mice received one dose of 0.5 mg/kg ACY-1215, 50 mg/kg ACY-1215, or 50 mg/kg ACY-1215 plus 0.5 mg/kg bortezomib for analysis of serum concentration of ACY-1215. For analysis of drug concentration in tumor tissue, ACY-1215 was administered at 50 mg/kg with or without bortezomib. (Continued on the following page.)
ACY-1215 concentrations after 50 mg/kg alone showed an increase when co-administered with bortezomib, respectively. Initially, ACY-1215 concentration declined rapidly as drug partitioned into the intratumoral compartment but subsequently exhibited a slower elimination with a terminal half-life of 5.0 hours. Apparent clearance of ACY-1215 was increased when co-administered with bortezomib (Fig. 3A) but no change occurred in PMBCs (Supplementary Fig. S1).

The effects of ACY-1215 on the UPR were enhanced by dual targeting of protein degradation pathways with simultaneous bortezomib exposure. Treatment with ACY-1215 and bortezomib at their IC50 for 48 hours led to a synergistic increase in polyubiquitinated proteins (Fig. 3B). The combination also led to greatly enhanced induction of key regulators of the UPR, including GRP78, PERK, p-eIF2α, p-IRE-1, and spliced XBP-1 (Fig. 3C). Selective targeting of HDAC6 inhibits aggresome-mediated protein degradation leading to the induction of the UPR. ACY-1215 also acts directly on GRP78 leading to the release of PERK and activation of the PERK apoptosis pathway (Fig. 3D).

ACY-1215 plus bortezomib led to significant tumor growth delay and prolonged OS in a xenograft model of DLBCL. The effects of ACY-1215 alone and with bortezomib were validated in an in vivo model of DLBCL. Mice were treated with ACY-1215 50 mg/kg intraperitoneally, bortezomib 0.5 mg/kg, or the combination. Treatment was well tolerated, with weight loss >10% observed in both the ACY-1215 alone and combination cohorts within the first 5 days but returned to baseline thereafter (Fig. 4A). There was one (1 of 9) toxic death in the ACY-1215 cohort but none in the combination cohort (0 of 10).

Following only one cycle of therapy, the combination led to a statistically significant tumor growth delay compared with single agents (P = 0.006) with a mean doubling time of 16.71 days compared with 10.68 days for mice treated with ACY-1215 alone and 10.42 days for mice treated with bortezomib alone. Mice treated with only 1 cycle of the combination demonstrated a statistically significant survival advantage (P < 0.05; Fig. 4C).

The concentration of ACY-1215 and bortezomib was measured at sequential time points in serum and tumor tissue after a single injection of ACY-1215 0.5 or 50 mg/kg or in combination with bortezomib (0.5 mg/kg; Fig. 5A–C). At 0.5 hours after administration of 0.5 or 50 mg/kg ACY-1215, the serum concentration was 56 and 10,888 nmol/L, respectively. The addition of bortezomib resulted in a lower ACY-1215 serum concentration after administration of 50 mg/kg with a level of 881 nmol/L. At 1 hour after administration, this difference disappeared (588 and 402 nmol/L for ACY-1215 alone or with bortezomib, respectively). Initially, ACY-1215 concentration declined rapidly as drug partitioned into the intratumoral compartment but subsequently exhibited a slower elimination with a terminal half-life of 5.0 hours. Apparent clearance of ACY-1215 was increased when co-administered with bortezomib (29.960 and 5631 ml/kg/h, respectively). Intratumoral ACY-1215 concentrations after 50 mg/kg alone showed an increase from 163 nmol/L at 1 hour after administration to 16,002 nmol/L at 4 hours followed by a decrease to 256 nmol/L at 8 hours (Fig. 5A and B). Six hours after administration of the combination, the ACY-1215 concentration was 493 nmol/L, suggesting an intratumoral concentration of ACY-1215 in the same range for the co-administration with bortezomib. Importantly, it was noted that drug concentrations measured in tumor tissue were higher than what was measured in serum, suggesting that the drug efficiently accumulates within the tumor. In addition, the concentrations achieved are similar or greater than the concentrations found to induce cytotoxicity in the in vitro experiments. In addition, these concentrations also recapitulate what has been described in human PK studies. Bortezomib 0.5 mg/kg reached a serum concentration of 7.5 nmol/L at 0.5 hours, after which the concentration decreased with a fluctuating pattern (Fig. 5C) similar to what has been reported previously (28).

Following a single treatment dose, tumor tissue was compared with spleen and untreated tumor tissue for modulation of α-tubulin, GRP78, XBP-1, and induction of apoptosis. Tumor tissue from mice treated with ACY-1215 and the combination demonstrated more intense staining for acetylated α-tubulin (+1 and +2, respectively) compared with no appreciable staining in the treated spleen or untreated tumor tissue (Fig. 5D). GRP78 and XBP-1 expression was induced in the tumor tissue treated with ACY-1215 (+2) and the combination (+1) compared with the treated spleen and untreated tumor tissue. After only 6 hours of a single exposure, apoptosis was observed in the tumor tissue under both treatment conditions. Interestingly, the effects of ACY-1215 appeared to be restricted to the tumor tissue as compared with the spleen suggesting selective activity for neoplastic lymphocytes. Acetylation of α-tubulin and induction of GRP78 were confirmed by Western blotting from mouse tumor tissue extract.

Key regulators of the UPR are upregulated in human lymphoma tissue

Patient samples were analyzed for HDAC6, GRP78, XBP-1, and CHOP by IHC (Fig. 6A). These samples comprised 43 DLBCLs, 13 follicular lymphomas (FL), 10 marginal zones (MZL), 8 MCLs, 13 TCLs of varying subtypes, 3 SLL/CLL, 2 posttransplant lymphoproliferative disorders (PTLD), and 14 reactive lymphoid tissues (total = 134; Fig. 6B). Forty-one lymphomas were assessed for HDAC6 staining, which was ubiquitously expressed in all subtypes as compared with reactive lymphoid tissue. Conversely, CHOP was not expressed at all in either lymphoma samples or reactive lymphoid tissue. Of the 82 lymphoma samples evaluable for GRP78, 80% (66 of 82) exhibited +1 or +2 staining compared with only 5% (1 of 19) of the reactive lymphoid samples (P < 0.0001). Comparison of aggressive lymphomas (DLBCL, MCL, Ki67 >30%, TCL, and PTLD) and indolent lymphomas (SLL/CLL, follicular lymphoma, MZL) revealed that the aggressive diseases were more likely to express GRP78 than the indolent subtypes (92% vs. 8%).

(Continued) C. Graphical representation of bortezomib concentration over time analyzed in serum and tumor tissue. Mice received one dose of 0.5 mg/kg bortezomib via intraperitoneal route. D. IHC staining of acetylated α-tubulin, GRP78, XBP-1, and TUNEL assay for apoptosis at 6 hours after a single injection of 50 mg/kg ACY-1215 with or without 0.5 mg/kg bortezomib. Treated tumor tissue was compared with the spleen of the same mouse and with untreated control mice tumor tissue. Immunoblot analysis of GRP78 and acetylated α-tubulin from whole-cell lysates of mouse tumor tissue treated with ACY-1215 alone or in combination with bortezomib. Mice were treated with a single intraperitoneal injection of ACY-1215 at 0.5 or 50 mg/kg and analyzed at 4 and 6 hours. Densitometry analysis was performed on scanned immunoblot images using the ImageJ software (NIH). Relative levels of acetylated α-tubulin protein expression were calculated and plotted.
50%, respectively; \( P < 0.0001 \)). Staining was more intense in aggressive lymphomas with 43% demonstrating \( + \) staining compared with 9% of indolent lymphomas \( (P = 0.004) \). Similar patterns of expression were observed for XBP-1 with 77% of the lymphoma samples demonstrating increased protein expression as compared with 5% of the reactive lymph nodes and tonsil samples \( (P < 0.0001) \). The aggressive lymphomas showed XBP-1 expression in 83% of evaluable samples compared with 63% of indolent samples \( (P = 0.039) \) and 33% of the aggressive subtypes had \(-2\) staining as compared with 4% of indolent lymphomas \( (P = 0.005) \). OS was significantly longer in patients with DLBCL who demonstrated staining intensity \( +2 \) of GRP78 than those with less intense staining \( +1; \ P = 0.0195; \) Fig. 6C). This finding appeared consistent across all lymphoma subtypes \( (P = 0.0315; \) Fig. 6D). Of the 24 patients with \textit{de novo} DLBCL who received
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CHOP chemotherapy, intense staining of GRP78 was correlated with a higher likelihood of obtaining a compete response and improved OS. Of those with a complete response, 70% had 2+ staining as compared with only 30% of patients who had relapsed or refractory disease or early death. Median OS of patients with 2+ GRP78 was 6.6 years compared with 0.7 years in patients with 1+ staining.

Discussion

It has been nearly 15 years as the first data emerged suggesting that targeting protein degradation pathways, that is the 26S proteasome, could be therapeutic in lymphoma (2, 3). While proteasome inhibitors are regarded as pleiotropic drugs, years of mechanistic studies have yielded substantial insight into our understanding of intracellular protein dynamics. Although the role of the aggresome in hematologic malignancies has only recently come to light, it is clear that dual targeting of these complementary protein degradation pathways can produce synergistic affects. It has been recognized for years that there is a class synergy between proteasome inhibitors and HDAC inhibitors, though the precise mechanism has been elusive (7, 34, 35).

The data presented here are the first to comprehensively establish that pharmacologic modulation of the aggresome pathway and UPR with an isoform-selective HDAC6 inhibitor is active across heterogeneous subtypes of lymphoma. Our work also demonstrates that HDAC6 and other mediators of the UPR are highly upregulated in primary neoplastic tissue compared with reactive lymphocytes, making this pathway an attractive therapeutic target for selective inhibition. The lymphomas represent a group of malignancies that can be characterized by their enhanced capacity to induce the UPR allowing neoplastic lymphocytes to maintain a high level of protein production while evading cell death under conditions of misfolded protein accumulation. Efforts to target the UPR in preclinical models of MCL with nonselective HDAC inhibitors and bortezomib have supported our findings (7, 34). Rao and colleagues demonstrated similar modulation of the UPR using panobinostat with bortezomib with less synergism and higher expression while evading cell death under conditions of misfolded protein accumulation. Efforts to target the UPR in preclinical models of MCL with nonselective HDAC inhibitors and bortezomib have supported our findings (7, 34). Rao and colleagues demonstrated similar modulation of the UPR using panobinostat with bortezomib with less synergism and higher probability for off-target effects.

Selective targeting of HDAC6 inhibits aggresome-mediated protein degradation leading to the induction of the UPR and apoptosis (Fig. 3D). Increasing exposure to ACY-1215 led to acetylation of α-tubulin which disrupted the binding of misfolded protein aggregates to the HDAC6–tubulin–motor dynein complex. This effect led to induction of apoptosis. In addition, inhibiting HDAC6 had a direct effect on GRP78, the master regulator of the UPR, which upon treatment became acetylated and released principle effectors of the UPR, including PERK, and likely ATF6, and IRE-1. As anticipated, dual targeting of protein degradation pathways with ACY-1215 and bortezomib led to profound synergism in both cell-based and in vivo studies. One hypothesis for this observation may be that the lymphomas are maximally compensating for a high misfolded protein burden as evidenced by a constitutively upregulated UPR. By interfering with both aggresome mediated- and ubiquitin–proteasome–mediated proteostasis, all alternative mechanisms for processing the large burden of accumulated misfolded proteins are obstructed, leading to overwhelming endoplasmic stress and a shift toward apoptosis. This hypothesis has been supported by several findings presented in above. Treatment of normal PBMCs with ACY-1215 alone or in combination with bortezomib demonstrated no effect on cytotoxicity or modulation of the UPR underscoring the notion that this strategy works best in stressed malignant lymphocytes with an overcompensating UPR. Interestingly, upon evaluation of the in vivo experiment, no pharmacodynamic changes were observed in the spleens of mice treated with ACY-1215 alone or in combination with bortezomib compared with malignant tissue. In addition, this hypothesis is supported by the evaluation of primary lymphoma samples that strongly express HDAC6 as compared with reactive lymphoid tissue. This strong expression was seen evenly with no difference in expression between different lymphoma subtypes. In addition, primary samples of aggressive lymphomas expressed increased GRP78 and XBP-1. Interestingly, patients with high expression of GRP78 were more likely to attain a complete response to CHOP-based chemotherapy and demonstrated a prolonged OS (P = 0.0195). The prognostic relevance of GRP78 in DLBCL has been evaluated in one previous publication (30). Mozos and colleagues evaluated patients with DLBCL treated with R-CHOP and demonstrated a worse prognosis with high GRP78 expression (P = 0.048). Despite these initial findings, the authors demonstrated in cell-based assays that this phenomenon could be overcome with the addition of bortezomib to R-CHOP, further supporting the notion that modulation of the UPR pathway results in significant cytotoxicity in DLBCL.

The dissimilarities in outcomes between our study and Mozos’ may be due to different patient populations and small numbers studied and certainly these associations need further study in larger populations. Evaluating the pharmacodynamic effects of ACY-1215 in a DLBCL mouse model confirmed the ability to modulate α-tubulin and induce apoptosis after a single injection of drug. A single administration of ACY-1215 achieved concentrations equal to/greater than the therapeutic range necessary for apoptosis in in vitro studies. ACY-1215 accumulated in the tumor tissue at higher concentrations than in the serum and corresponded with higher levels of acetylated α-tubulin, GRP78, and apoptosis as compared with paired splenic tissue suggesting a selectivity for neoplastic lymphocytes.

While the recent emphasis on new targeted treatments for lymphoid malignancies has been focused on inhibiting downstream targets of the B-cell receptor pathway, targeting ER stress and the UPR are time-honored treatment strategies. The data presented herein demonstrate that targeting protein degradation pathways also fits the paradigm of precision targeting of critical pathogenic pathways, which is underscored by the observed differences seen between normal and malignant lymphocytes. Interestingly, the effects of HDAC6 inhibition do not separate by cell of origin in DLBCL. These data are the first to establish the merits of targeting this biology in preclinical models and to correlate these findings directly with patient-derived tissue. It is clear that our improved understanding of intracellular protein dynamics is creating a logic that is informing novel combinations with drugs like proteasome and HDAC inhibitors. Future studies will be focused on evaluating levels of GRP78 as a biomarker for response to ACY-1215 in a clinical trial of ACY-1215 in patients with relapsed or refractory lymphoma (NCT02091063).

Disclosure of Potential Conflicts of Interest

J.E. Amengual reports receiving commercial research grants from and is a consultant/advisory board member for a Acetylon Pharmaceuticals, Inc. S. Jones has ownership interest (including patents) in Acetylon Pharmaceuticals.

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