Inhibition of histone deacetylase overcomes rapamycin-mediated resistance in diffuse large B-cell lymphoma by inhibiting Akt signaling through mTORC2

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The mammalian target of rapamycin (mTOR) has emerged as an important therapeutic target for diffuse large B-cell lymphoma (DLBCL), as recent studies have demonstrated that 30% of relapsed patients respond to mTOR inhibitors. Why some lymphomas are resistant is incompletely understood. In the present study, we demonstrated that rapamycin inhibits mTORC1 in DLBCL lines and primary tumors but is minimally cytotoxic. Subsequent investigations revealed that rapamycin also activated eIF4E and the mTORC2 target Akt, suggesting a potential mechanism of rapamycin resistance. Furthermore, knockdown of the mTORC2 component rictor, but not the mTORC1 component raptor, inhibited rapamycin-induced Akt phosphorylation in lymphoma cells. Addition of the histone deacetylase inhibitor (HDi) LBH589 (LBH) overcame rapamycin resistance by blocking mTOR, thus preventing Akt activation. Further studies support the involvement of the protein phosphatase P1 in LBH-mediated Akt dephosphorylation, which could be mimicked by knockdown of HDAC3. This is the first demonstration that a HDi such as LBH can overcome rapamycin resistance through a phosphatase that antagonizes mTORC2 activation. These results provide a mechanistic rationale for a clinical trial of a combination of HDi and mTOR inhibitors for DLBCL. (Blood. 2009;114: 2926-2935)

Introduction

Diffuse large B-cell lymphoma (DLBCL), an aggressive form of non-Hodgkin lymphoma (NHL), is the most common type of lymphoma in the United States. With rituximab-based chemotherapy, such as rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, approximately 60% of DLBCL patients are cured.1,2 Salvage chemotherapy followed by stem cell transplantation is able to produce durable remissions in a minority of relapsed patients, and improved therapy is required for those who relapse after second-line treatment.

Because deregulation of the PI3 kinase (PI3K)/mTOR pathway occurs in many human diseases,3,4 targeting the mTOR pathway with small molecule inhibitors has become an important area of research. Key components of this pathway, including Akt and mTOR, regulate cell growth and survival.5 The mTOR kinase exists as 2 complexes. The rapamycin-sensitive mTOR complex 1 (mTORC1 or raptor/mTOR), consists of mTOR, raptor, and mLST8. mTORC1 regulates translation initiation through 2 distinct pathways: ribosomal p70 S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs). In one pathway, mTORC1 phosphorylates and activates the ribosomal protein S6. In the second pathway, mTORC1 directly phosphorylates 4E-BP1 causing its dissociation from the translation initiation factor eIF4E. This allows eIF4E to stimulate cap-dependent RNA translation. In the absence of mTORC1 activation, 4EBP1 binds tightly to eIF4E, preventing it from binding to 5’-capped mRNA.6 The mTOR complex 2 (mTORC2 or rictor/mTOR), which contains mTOR, rictor, and mLST8, is rapamycin insensitive and functions to regulate the survival kinase Akt by phosphorylation of serine 473.5

Recent clinical trials of the mTORC1 inhibitors temsirolimus and everolimus, both analogues of the parent compound rapamycin, have demonstrated overall response rates (ORRs) of approximately 30% for relapsed DLBCL.7 This single-agent activity of mTOR inhibitors in heavily pretreated DLBCL patients highlights the importance of the PI3K/mTOR pathway in these cells. To exploit the sensitivity of lymphomas to mTOR inhibitors through effective therapies, it is important to understand the mechanistic basis for resistance of DLBCL to mTOR inhibition.

Histone deacetylase inhibitors (HDIs) have emerged as a potentially promising new class of anticancer drugs. The inhibition of histone deacetylases (HDACs) by HDIs results in increased gene-specific histone acetylation, which can lead to reactivation of silenced genes, morphologic reversion of transformed cells, differentiation, inhibition of cell growth, induction of apoptosis, and inhibition of angiogenesis in cancer cell lines.8,9 Several structurally diverse classes of synthetic compounds have been identified as HDIs.10,11 HDACs are involved in the pathogenesis of some lymphomas, notably cutaneous T-cell lymphoma.12 Vorinostat, a potent oral HDI belonging to the class of hydroxamic acid-containing hybrid polar molecules, is now FDA approved for relapsed cutaneous T-cell lymphoma.13 The potential role of HDACs in other lymphoma is not well understood.

LBH589 (LBH) is a cinnamic acid hydroxamate HDI currently being tested in clinical trials for various malignancies. LBH inhibits cell proliferation and induces apoptosis in preclinical models. Moreover, LBH exhibits antileukemic effects in phase 1 studies.14 The goals of the current studies were to investigate the mechanisms of resistance of DLBCL to mTOR inhibition and to determine whether a HDI such as LBH can overcome this
resistance. Our results demonstrate that important and unanticipated effects of LBH on mTORC1/mTORC2 signaling in DLBCL cells lead to synergistic inhibition of lymphoma cell survival when rapamycin and LBH are combined.

Methods

Reagents

LBH589 (panobinostat) was provided by Novartis-Pharmaceutical. Annexin V-fluorescein isothiocyanate was obtained from BD Biosciences. Rapamycin and antibodies (total and phospho) specific for p85, PDK1, Akt (Ser 473), S6 (Ser 235/236), mTOR (Ser 2448), 4E-BP1 (Thr 37/46), eIF4E (Ser 219), rictor, raptor, H3, H4, and all the HDACs were obtained from Cell Signaling Technology. Antibodies for protein phosphatase 1 (PP1) and PP1 alpha (PP1a) were from Santa Cruz Biotechnology. Calcyulin A and ZVAD-fmk were purchased from Calbiochem and R&D Systems, respectively. PI3K inhibitors LY294002 and wortmannin were purchased from Calbiochem. Akt-specific inhibitor A443654 was a kind gift from S.H.K.

Lymphoma samples

Tumor samples from DLBCL were obtained through the University of Iowa/Mayo Lymphoma SPAORE Biospecimens Core. All samples were surgical waste, and patients signed informed consent to provide excess tissue for research on a protocol approved by the Mayo Clinic institutional review board, in accordance with the Declaration of Helsinki. Mononuclear cells were isolated from lymph node biopsies by mechanical disruption over a wire mesh screen followed by Ficoll-Hypaque centrifugation. A total of 20 primary samples of DLBCL were used for this study. SU DH6 (DHL-6), O CI-Ly7 (Ly7), and OCT-Ly3 (Ly3) DLBCL cell lines were a gift from Dr Margaret Shipp (Dana-Farber Cancer Center). Ly7 and Ly3 cell lines were cultured in IMDM medium supplemented with 10% fetal calf bovine serum. DHL-6 was maintained in RPMI with 10% fetal calf bovine serum.

Immunohistochemistry

Sections (4 μm thick) were cut from formalin-fixed paraffin-embedded tissue blocks. After drying at 60°C for 60 minutes, the slides were deparaffinized. Endogenous peroxidase activity was quenched by incubation of sections in a 1:1 mixture of 3% hydrogen peroxide and absolute methanol. The slides were stained using DAKO autostainer under standard conditions, rinsed well, counterstained with hematoxylin for 30 seconds, and mounted with aqueous mounting media. All slides were observed with light microscopy (Olympus AX70, 400×/0.75 numeric aperture; Olympus America). All images were captured with SPOT RT camera and software (Diagnostic Instruments).

Cell survival by annexin V/PI

Malignant cells from DLBCL patients (0.5 × 10⁶ cells/mL) or from DLBCL lines were incubated in 48-well plates (0.5 mL/well). Cells were cultured in the presence of various concentrations of LBH or rapamycin at 37°C, stained using annexin V–fluorescein isothiocyanate and propidium iodide (PI), and analyzed by flow cytometry (FACSCalibur; Becton Dickinson). Data analysis was performed with FlowJo software (TreeStar).

Proliferation assay

Cells from DLBCL lines were cultured for 48 hours at 37°C in 96-well round-bottom microtiter plates (Costar) at a density of 5 × 10⁴ cells/well in the presence of various concentrations of LBH. Before harvesting, cells were pulsed with 1 μCi (0.037 MBq) tritiated thymidine (³H-TdR; American) for 18 hours. ³H-TdR incorporation levels were determined using a Beckman scintillation counter (GMI).

Western blot analysis

Cells (5 × 10⁶) were incubated with various concentrations of LBH or rapamycin at 37°C for various lengths of time, as indicated. Cells were washed with cold PBS and lysed with radioimmunoprecipitation assay lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Roche Diagnostic). After clarification of the lysates by centrifugation, proteins were resolved on 10% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked and probed with antibodies as indicated and subsequently incubated with peroxidase-conjugated secondary antibody. Bound antibodies were visualized using Pierce Super Signal (Amersham Biosciences). After incubation at room temperature for 15 minutes in stripping buffer (Pierce Biotechnology), blots were reprobed with additional antibodies.

Coimmunoprecipitation assay

To the cleared lysates, 5 μg specific antibody was added and complexes were allowed to form by incubating with rotation for 60 minutes at 4°C. A 50% slurry (25 μL) of protein A–Sepharose was then added and the incubation continued for 1 hour. Immunoprecipitates captured with protein A–Sepharose were washed 4 times with radioimmunoprecipitation assay buffer and then analyzed by immunoblotting.

siRNA transfection

DHL-6 and Ly7 cells were transfected with human B cell Nucleofector kit (Amaxa Biosystems). Briefly, 10 × 10⁶ cells were transiently transfected with 250 nM or 500 nM rictor or raptor (Dharmacon) or HDAC3 or HDAC4 siRNA (Santa Cruz Biotechnology) using U-15 program. For c-Myc inhibition, c-Myc siRNA kit (Cell Signaling Technologies) was used according to the supplier’s instructions.

Results

Activation of the mTOR signaling pathway in lymphoma samples from DLBCL patients

To examine activation of the mTOR pathway in fresh human lymphoma tissues, immunohistochemistry was performed on primary tumor samples from 10 DLBCL patients using antibodies against mTOR and its downstream targets the ribosomal protein S6 (S6rp) and 4EBP1. Phosphorylation of mTOR, S6, 4EBP1, and Akt was detected in all 10 lymphomas (Figure 1A).

DLBCL cells are resistant to rapamycin cytotoxicity

The finding that the mTOR signaling pathway is constitutively activated in primary DLBCL tissue suggested that mTOR inhibition might inhibit growth of these cells. Three representative DLBCL cell lines, DHL-6, Ly7, and Ly3, were treated with increasing doses of rapamycin (5 nM-100 nM) for 48 hours and assessed for the effects on proliferation and survival. These concentrations of rapamycin were chosen based on achievable concentrations of rapamycin in humans. Rapamycin produced less than a 10% inhibition of cell survival in all cell lines without evidence of a dose-dependent effect (Figure 1B-D). Because DLBCL lines differ from fresh primary DLBCL samples in terms of growth rates, altered microenvironment, and the absence of interacting stroma, we treated malignant cells from 3 different DLBCL patients with rapamycin in vitro and assessed lymphoma cell survival at 48 hours. As observed in the cell lines, the survival of the primary DLBCL cells was minimally reduced in response to rapamycin (Figure 1E).

Although rapamycin does not affect lymphoma cell survival, we did demonstrate partial inhibition of proliferation in all of the DLBCL lines. This was not, however, dose dependent (supplemental Figure 1A-B, available on the Blood website; see the Supplemental Materials link at the top of the online article). These results
suggest that lymphoma cells may have a potential mechanism to bypass growth inhibition caused by rapamycin.

**Inhibition of mTOR by rapamycin increases the target of mTORC2 while decreasing mTORC1 targets**

The results in Figure 1 prompted us to ask whether rapamycin was actually blocking mTOR and how rapamycin differentially affected mTORC1 versus mTORC2 in DLBCL cells. The effects of rapamycin on mTORC2 signaling in DLBCL were investigated by examining the mTORC2-mediated phosphorylation of Akt on Ser 473. Unexpectedly, both cell lines exposed to rapamycin exhibited a dose- and time-dependent increase in Akt phosphorylation (Figure 2A-B). In addition, the phosphorylation of eIF4E, a molecule downstream of mTORC1 that is involved in cell growth (Figure 2A-B), was also increased. mTOR inhibition by rapamycin would be expected to inhibit eIF4E phosphorylation (via dephosphorylation of 4E-BP1) rather than stimulate eIF4E phosphorylation. How mTOR inhibition activates eIF4E phosphorylation remains unclear. It has been demonstrated that the MEK/ERK and p38 MAPK signaling pathways activate eIF4E through Mnk1-mediated phosphorylation of eIF4E. Further studies are ongoing to investigate the role of Mnk1 in rapamycin-induced eIF4E phosphorylation. To correlate the dynamic changes in phospho-Akt and phospho-eIF4E with the inhibition of mTOR in response to rapamycin, we examined the effects of rapamycin dose and exposure time. Phosphorylation of Akt and eIF4E increased soon after a 4-hour exposure to rapamycin and was sustained for up to 24 hours (Figure 2A). Primary DLBCL
samples exposed to rapamycin in vitro also contained elevated levels of p-Akt and p-eIF4E (Figure 2C).

After treatment with rapamycin, the phosphorylation of S6rp, 4EBP1, and mTOR was reduced in a time- and dose-dependent manner (Figure 2A-B), confirming the ability of rapamycin to inhibit mTORC1 signaling. However rapamycin reduced pS6rp more effectively than p4EBP1. Likewise, rapamycin diminished the phosphorylation of S6 in all DLBCL samples examined (n = 4; Figure 2C). Thus, despite a lack of cytotoxicity, mTORC1 is indeed being inhibited by rapamycin in DLBCL cells. Taken together, our results suggest that rapamycin triggers a negative feedback mechanism by activating survival pathways involving Akt and eIF4E that may attenuate the antiproliferative effects mediated through mTORC1.

**mTOR inhibition also causes increased Akt signaling in malignant lymphoma cells in vivo**

To further explore rapamycin-induced Akt phosphorylation, we examined peripheral blood samples from patients with relapsed aggressive B-cell NHL who were treated with single-agent temsirolimus on a clinical trial.

Phosphorylation of Akt and eIF4E was detectable at baseline and was increased in each of the 3 patient samples at 24 hours, at 48 hours, and after 4 weekly doses (1 cycle) of temsirolimus (Figure 2D). Due to the unavailability of additional temsirolimus-treated patient samples, instead of probing with total Akt or eIF4E, we probed the same blot for p-S6 to determine the status of mTORC1 in the same samples. These results provide the first evidence that rapamycin derivatives such as temsirolimus are capable of increasing Akt signaling in patients treated with these inhibitors.

**The HDAC inhibitor LBH inhibits the survival and proliferation of lymphoma cells by inhibiting HDAC3 and HDAC4 expression**

In a parallel set of experiments, we investigated whether the HDI LBH would inhibit proliferation and survival of DLBCL cells. To verify that LBH inhibits its targets in these cells, the DHL-6, Ly7, and Ly3 lines were treated with increasing LBH concentrations and assayed by immunoblotting for changes in histone acetylation.
expected, there was a progressive increase in acetylated histones H4 and H3 (Figure 3A). Further analysis demonstrated that increasing doses of LBH (5-100 nM) also suppressed the survival of each cell line in a dose-dependent manner at 48 hours (Figure 3B-C). IC50 values for these cell lines were 20 nM (DHL-6) and 10 nM (Ly7). Subsequent testing showed that all DLBCL clinical specimens (n = 3) were also sensitive to LBH, even at the lowest dose (10 nM) of the drug (Figure 3D). Pretreatment of LY7 cells with a caspase inhibitor Z-VAD-fmk did not prevent LBH-induced cell death (Figure 4E). These results suggest that LBH-induced apoptosis of DLBCL cells involves a caspase-independent mechanism. LBH also inhibited 3H-thymidine incorporation in a concentration-dependent manner (Figure 3F-G).

HDACs (supplemental Figure 2). These data suggest that HDAC3 and HDAC4 may be important to the growth of DLBCL.

Rapamycin and LBH synergistically inhibit survival and proliferation of DLBCL cells

Combined treatment of DHL-6 cells with 5, 10, or 20 nM rapamycin with the same concentrations of LBH for 48 hours significantly reduced the survival of DHL-6 cells (Figure 4A). To determine whether this shift in IC50 for the combinations reflected drug synergy, we calculated the combination index (CI) using the Chou-Talalay equation.18 Formal mathematic analysis indicated that rapamycin and LBH were synergistic, as indicated by CI values of less than 1.0 (ie, CI was 0.5 at 5 nM, 0.8 at 10 nM, and 0.9 at 20 nM). Similarly, cotreatment of Ly7 cells with 2.5, 5.0, or 10 nM rapamycin with the same doses of LBH for 48 hours also significantly decreased the survival of Ly7 cells (Figure 4B). The CI values for Ly7 cells were more synergistic than for DHL-6 cells (ie, CI was 0.1 at 2.5 nM, 0.3 at 5 nM, and 0.4 at 10 nM). The LBH/rapamycin combination also reduced proliferation of DHL-6 and Ly7 cells more effectively than either agent alone (Figure 4C-D).

LBH effectively targets mTORC1 signaling

In experiments designed to determine the effect of LBH on the mTORC1 targets S6 and 4EBP1, we demonstrated that single-agent LBH was able to inhibit the phosphorylation of S6 and 4EBP1 along with mTOR in a time- and dose-dependent manner (Figure 5A-B). LBH also inhibited the phosphorylation of S6 in all 4 DLBCL patient samples examined (Figure 5C). Next, we determined the effects of cotreatment with LBH and rapamycin on the mTORC1 signaling proteins. Doses of LBH and rapamycin that were noninhibitory as single agents were in combination able to produce more inhibition of the phosphorylation of S6rp and 4EBP1 in DHL-6 cells than treatment with either agent alone (Figure 5D).
LBH inhibits constitutive as well as rapamycin-induced Akt (mTORC2) phosphorylation

Because our previous experiments demonstrated that one compensatory effect of rapamycin treatment in DLBCL cells was phosphorylation of Akt, we investigated the effect of LBH on Akt. LBH caused a dose-dependent decrease in the Ser 473 phosphorylation of Akt in DHL-6 and Ly7 cells without changing the level of total Akt (Figure 5E). In addition to the effects on Akt, LBH also inhibited the phosphorylation of eIF4E in a time- and dose-dependent manner (Figure 5E-F). Similar effects of LBH on the phosphorylation of Akt and eIF4E were observed in samples from DLBCL patients (n = 4; Figure 5G). Rapamycin treatment alone increased Akt and eIF4E phosphorylation levels in both DLBCL lines. This effect on p-Akt could be effectively blocked if LBH was added with the rapamycin (Figure 5H).

Mechanism of LBH-mediated Akt dephosphorylation

Potential mechanisms of LBH-mediated Akt dephosphorylation are through the deactivation of upstream proteins or the activation of an Akt-specific phosphatase. We treated DHL-6 cells with LBH and assessed the expression levels of a series of signaling proteins related to the regulation of Akt signaling, including the p85 regulatory subunit of PI3K and phosphoinositide-dependent protein kinase 1 (PDK-1). As shown in Figure 6A, LBH exposure did not alter the expression or phosphorylation of either of these signaling proteins. The reported ability of HDAC inhibitors to disrupt HDAC-PP1 complex formation led us to investigate whether LBH-induced alterations in PP1 could contribute to the observed effects on Akt phosphorylation. We first examined the possibility that LBH might be increasing phosphatase expression, but observed that LBH did not increase the total levels of PP1 and PP1A (Figure 6B). Instead, immunoprecipitation experiments revealed that LBH treatment resulted in increased binding of PP1 to Akt (Figure 6B). To determine whether this increased binding of PP1 to Akt might be contributing to increased Akt phosphorylation, we examined the effects of LBH on Akt phosphorylation in the absence or presence of the PP1 inhibitor calyculin A. As indicated in Figure 6C, calyculin A diminished the effect of LBH on Akt phosphorylation. To validate the role of HDAC3 and HDAC4 in Akt deactivation, we used siRNAs to selectively knock down the expression of HDAC3 and HDAC4 and a control siRNA as a negative control (supplemental Figure 3A). We showed that repression of HDAC3 but not HDAC4 led to decreased Akt phosphorylation, whereas control siRNA transfection had no effect on phospho-Akt levels (Figure 6D). The effects of LBH on Akt are clear, but to better understand whether this blockage of Akt contributes to LBH-induced apoptosis, DHL-6 cells were transiently transfected with constitutively active Akt (CA-Akt). Significant protection from cell death was observed in CA-Akt–expressing cells after treatment with LBH alone or LBH plus rapamycin (Figure 6E). The function of the CA-Akt construct was monitored by enhancement of p-Akt (Figure 6F).

LBH alters the level of intact mTOR2

To determine the effect of LBH on mTORC2, we treated Ly7 cells with LBH and/or rapamycin for 24 hours and compared the amounts of raptor and rictor bound to mTOR. Rapamycin had
little effect on the expression levels of mTOR, raptor, or rictor, but as expected, strongly reduced the amounts of raptor recovered with mTOR (Figure 6G) and increased the amount of rictor bound to mTOR (Figure 6H). LBH, on the other hand, diminished the amount of rictor or raptor bound to mTOR (Figure 6G-H).

To confirm the importance of mTORC2 but not mTORC1 in the rapamycin-induced Akt phosphorylation, we used small interfering RNA (siRNA) to specifically knock down rictor or raptor (supplemental Figure 3B). As indicated in Figure 7A, knockdown of rictor but not raptor prevented rapamycin-induced phosphorylation of Akt on Ser 473. These results demonstrate that mTORC2 complexes are responsible, at least in part, for the Akt Ser 473 phosphorylation observed upon treatment with the rapamycin. Next, we asked whether inhibition of rictor-mediated signaling could increase the survival of rapamycin-treated DLBCL cells. Rictor or raptor knockdown cells were treated with rapamycin for 48 hours and stained with annexin V/PI to assess survival. As indicated in Figure 7B, down-regulation of rictor but not raptor partially increased the rapamycin-induced sensitivity in DHL-6 cells.

To investigate whether the PI3K (upstream of Akt) was playing any role in rapamycin-induced Akt activation, we blocked the PI3K activity with a PI3K-specific inhibitor (LY294002). We found that LY294002 had minimal effect on rapamycin-induced Akt phosphorylation at AktSer473 with no effect at the AktThr308 site (supplemental Figure 4A). Furthermore, treatment of Ly7 DLBCL cells with a specific Akt inhibitor (A443654) inhibited survival in a dose-dependent manner similar to LBH. Combining A443654 with rapamycin showed significant growth-inhibitory effects (supplemental Figure 4B-C). This again confirms that LBH exerts its cytotoxic effects in part through Akt.

**Combined treatment of LBH589 and rapamycin effectively targets c-Myc in DLBCL cells**

The mTOR pathway is considered to control the translation of specific mRNA transcripts, some of which are involved in cell-cycle control (eg, cyclin D1 and c-Myc). LBH alone resulted in a dose-dependent decrease in the expression of c-Myc in the Ly7 line, whereas cyclin D1 was not inhibited in either line (Figure 7C). In contrast, rapamycin treatment inhibited cyclin D1 expression, but only after 24 hours at high concentrations (50 nM, 100 nM). Rapamycin failed to inhibit the level of c-Myc in Ly7 cells at any dose (Figure 7C). Importantly, the combination of both rapamycin and LBH attenuated both cyclin D1 and c-Myc levels in Ly7 cells (Figure 7D). To better understand whether the inhibitory effects of LBH on proliferation are mediated through c-Myc downregulation, we used c-Myc siRNA to inhibit c-Myc expression in DHL-6 cells and demonstrated growth inhibition (Figure 7E-F). We further showed that inhibition of HDAC3 inhibited the c-Myc expression in DHL-6 cells, whereas it has little to no effect on cyclin D1 expression (Figure 7G).
Discussion

The PI3K/Akt/mTOR pathway has become an important focus for cancer therapeutics.21,22 The mTOR inhibitory agents that have been most thoroughly investigated to date as single agents are sirolimus (rapamycin), temsirolimus, and everolimus. Temsirolimus and everolimus are now approved by the FDA for relapsed renal cell carcinoma.23 mTOR inhibitors have also demonstrated impressive single-agent activity in relapsed lymphoma. Weekly intravenous temsirolimus produced an ORR of 38% and 41% in 2 studies of patients with relapsed mantle cell NHL.17,24 Additional trials of temsirolimus have produced similar results in relapsed follicular NHL and DLBCL.25 Preliminary results using oral everolimus have demonstrated that approximately 30% of patients with relapsed DLBCL respond; the ORR is higher, at 40%, for MCL, Waldenström macroglobulinemia,26 and Hodgkin disease.27,28 These single-agent trials have provided proof-of-concept that mTOR inhibition can produce antitumor responses in relapsed lymphoma.

Analyses of human DLBCL tumor specimens provide support that mTOR signaling pathways are frequently activated in DLBCL tumors, and others have shown that the expression of p-Akt is an adverse prognostic feature.29 Although rapamycin at low concentrations (even at 5 nM) can suppress the phosphorylation of S6rp (indicating that mTOR signaling has indeed been blocked), this results in only a modest antiproliferative effect, with minimal cytotoxicity in DLBCL lines and primary cells from DLBCL patients. This rapamycin resistance is consistent with the previous reports in DLBCL cell lines and other cancers.30,31 In the studies reported herein, we sought to understand the mechanism(s) of mTOR resistance in lymphoma and to use this information in the design of new treatment strategies that could overcome mTOR inhibitor resistance in lymphoma. We found that the mTORC2 target p-Akt was increased in DLBCL cell lines and primary samples after rapamycin exposure. Moreover, we detected significantly increased levels of p-Akt in lymphoma samples from patients treated with intravenous temsirolimus, consistent with the findings of Wang et al32 in lung cancer cell lines. It has been suggested that an equilibrium may exist between mTORC1 and mTORC2 complexes33; therefore, it is possible that inhibition of mTORC1 by an mTOR inhibitor shifts the equilibrium to favor activation of mTORC2, leading to an increase in Akt phosphorylation.34 Sarbassov et al34 reported that prolonged rapamycin treatment in cell types (other than lymphoma) could inhibit the assembly of mTORC2. However in our studies, prolonged treatment of lymphoma cells with high doses of rapamycin only slightly inhibited the Akt activation and did not completely abolish the p-Akt activation (data not shown). In addition, the PI3K inhibitors LY294002 and wortmannin were unable to abrogate rapamycin-induced Akt activation or to inhibit the survival of lymphoma cells. This suggests that PI3K activity is not required for Akt activation by rapamycin. Our data further show that the mTOR-rictor (TORC2) complex can directly phosphorylate Akt at Ser 473. This was demonstrated by our finding that disruption of mTORC2 by knocking down rictor blocked the rapamycin-induced Akt activation. In addition, mTORC1 knockdown with raptor siRNA did not block rapamycin-induced Akt phosphorylation in DLBCL cells. This finding is in contrast with the studies in lung cancer cells where rapamycin increased Akt phosphorylation independent of mTORC232 and consistent with the results of others in different
model systems. This suggests that rapamycin-induced Akt activation might be tissue and dose specific.

We showed that increased Akt phosphorylation in response to rapamycin most likely contributes to the attenuated antitumor activity of rapamycin. This resistance mechanism can be overcome in vitro in DLBCL cells with the pan-DAC inhibitor LBH589. LBH treatment also inhibits the constitutive activation of Akt in DLBCL. Other studies with another HDI (SAHA) found inhibition of Akt activity in prostate cancer cells and mantle cell lymphoma. These observations suggest that the Akt signaling pathway might be a common target of HDI in cancer cells. In our experiments, the LBH-induced dephosphorylation of Akt was not mediated through regulators upstream of Akt (PI3K kinase) but rather by activation of the protein phosphatase PP1 that subsequently inactivates Akt by dephosphorylation. These results are consistent with the results of Chen et al. Key differences between their results and ours were the use of a different HDI (trichostatin A vs LBH), different tumors (glioma and prostate cell lines vs lymphoma cell lines and primary samples), and different HDACs (HDAC1 and HDAC6 vs HDAC3) in our report.

Other key findings of our studies are the constitutive expression of activated eIF4E in DLBCL, the further increase in eIF4E with rapamycin treatment, and the ability of LBH to prevent this eIF4E activation in vitro. eIF4E expression in primary lymphoma samples (including DLBCL) has been demonstrated by others, and the increase in eIF4E with rapamycin has also been described in lung cancer cell lines. Because Akt and eIF4E are often associated with cell survival and resistance to cancer therapy, our findings imply that the activation of Akt and eIF4E through mTOR inhibition may counteract the anticancer efficacy of mTOR inhibitors. Our coimmunoprecipitation data suggest that LBH alone was able to alter the level of intact mTORC2/mTORC1 by reducing the amounts of rictor or raptor bound to mTOR, which is further decreased when combined with rapamycin. We have also seen that LBH alone was able to inhibit the activation of mTORC1 targets S6rp and 4E-BP1, whereas combined treatment with rapamycin inhibited the phosphorylation of S6rp and 4E-BP1 to a greater extent than either agent alone. In support of this model, rapamycin combined with LBH exhibited enhanced synergistic inhibitory effects on survival and proliferation of DLBCL cells in a dose-dependent manner along with induction of histone H3 and H4 acetylation. It is important to note that only nanomolar doses of each agent were needed for a potent synergistic cytotoxic effect on DLBCL cells. These doses should be achievable in humans.

An important function of mTOR is to phosphorylate S6rp and 4E-BP-1, resulting in the translation of mRNAs coding for proteins involved in cell-cycle regulation such as cyclin D1 and c-Myc. Deregulation of c-Myc proto-oncogene is implicated in the development of numerous human tumors including lymphoma. When overexpressed, c-Myc can trigger proliferation by driving quiescent cells into S-phase in the absence of other mitogenic stimuli. We have shown that all the DLBCL lines overexpress c-Myc protein. Surprisingly, LBH alone and in combination with rapamycin at very low doses was able to completely inhibit c-Myc expression and suppress cellular proliferation.

Our findings highlight the value of pathway-targeted combination therapy to achieve maximal blockade of signaling pathways for cancer treatment. These studies demonstrate for the first time to the best of our knowledge that pan-HDAC inhibitors such as LBH can actually interfere with the mTOR pathway both at the mTORC1 and mTORC2 level and overcome mTOR inhibitor resistance in DLBCL. Our data indicate that LBH overcomes rapamycin resistance by inhibiting Akt activation through 2 mechanisms, (1) through activation of protein phosphatase PP1 and (2) by inhibition of mTORC2 (mTOR-rictor), and not by inhibition of PI3 kinase. Based on these mechanistic preclinical studies, we propose that combination therapy with pan-HDAC and mTOR inhibitors targeting both mTORC1 and mTORC2 may be applicable to a broad spectrum of lymphoma patients, particularly those with aggressive disease.

Acknowledgments
This work was supported in part by Iowa/Mayo SPORE CA97724; R01CA127433; R21CA112904 (T.E.W.); and Iowa/Mayo SPORE (CA097274-07DRP6; M.G.).

Authorship
Contribution: M.G. designed and performed research, analyzed and interpreted data, and wrote the paper; S.M.A. and S.K. provided patient specimens and participated in editing the paper; A.J.N. assisted in interpretation of data and editing of the paper; S.H.K. designed research, interpreted data, and wrote the paper; and T.E.W. designed research, provided patient specimens, interpreted data, and wrote and edited the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


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