Dual HDAC and PI3K Inhibitor CUDC-907 Downregulates MYC and Suppresses Growth of MYC-dependent Cancers

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Abstract

Upregulation of MYC is a common driver event in human cancers, and some tumors depend on MYC to maintain transcriptional programs that promote cell growth and proliferation. Preclinical studies have suggested that individually targeting upstream regulators of MYC, such as histone deacetylases (HDAC) and phosphoinositide 3-kinases (PI3K), can reduce MYC protein levels and suppress the growth of MYC-driven cancers. Synergy between HDAC and PI3K inhibition in inducing cancer cell death has also been reported, but the involvement of MYC regulation is unclear. In this study, we demonstrated that HDAC and PI3K inhibition synergistically downregulates MYC protein levels and induces apoptosis in "double-hit" (DH) diffuse large B-cell lymphoma (DLBCL) cells. Furthermore, CUDC-907, a small-molecule dual-acting inhibitor of both class I and II HDACs and class I PI3Ks, effectively suppresses the growth and survival of MYC-altered or MYC-dependent cancer cells, such as DH DLBCL and BRD–NUT fusion-positive NUT midline carcinoma (NMC) cells, and MYC protein downregulation is an early event induced by CUDC-907 treatment. Consistently, the antitumor activity of CUDC-907 against multiple MYC-driven cancer types was also demonstrated in animal models, including DLBCL and NMC xenograft models. Myc transgenic tumor syngeneic models, and MYC-amplified solid tumor patient-derived xenograft (PDx) models. Our findings suggest that dual function HDAC and PI3K inhibitor CUDC-907 is an effective agent targeting MYC and thus may be developed as potential therapy for MYC-dependent cancers. Mol Cancer Ther; 16(2): 285–99. ©2016 AACR.

Introduction

MYC, one of the most frequently deregulated oncogenes in human cancer, encodes a transcription factor that plays a central role in tumor initiation and maintenance by orchestrating broad transcriptional changes that facilitate growth and proliferation (1). Tumors can employ multiple genetic and epigenetic mechanisms to upregulate MYC and commonly become dependent on MYC for proliferation and survival. Activating genetic alterations of MYC, including genomic rearrangements, copy number gains, or amplifications, are common driving events in certain cancers. For example, acquisition of MYC alterations and subsequent upregulation of MYC and MYC target genes have been implicated as key events in the transformation of indolent follicular lymphoma (FL) to highly aggressive diffuse large B-cell lymphoma (DLBCL) and is also observed in de novo DLBCL (2, 3). Alternatively, other oncogenic events drive cancer by inducing epigenetic changes that promote MYC overexpression such as in NUT midline carcinoma (NMC), a poorly differentiated, highly aggressive subtype of squamous cell carcinoma that primarily arises in midline organs (4). Approximately 80% of NMCs harbor a chromosomal translocation that fuses NUT to BRD3 or BRD4, which encode bromodomain and extraterminal domain (BET) proteins that regulate gene expression in part through the sequestration of p300 histone acetyltransferase activity and other transcriptional machinery to specific genomic loci (5). MYC is a major target of BET proteins, and NMC cells harboring BRD4–NUT fusions are dependent on MYC for maintenance of an undifferentiated, proliferative state (6, 7). Amplification or overexpression of the MYC oncogene is also associated with poor prognosis in other solid tumor types, such as medulloblastoma (8, 9), breast cancer (10), ovarian cancer (11), prostate cancer (12), and lung cancer (13).

Although MYC is a noncatalytic transcriptional regulator that has thus far proven undruggable, increasing evidence suggests that targeting upstream MYC regulators may be an effective strategy to suppress MYC. For example, HDAC inhibitors have been shown to suppress MYC transcription, in part by upregulating the transcription of MYC suppressor genes, such as FOXO1, in some (14–17) but not all cancer types (18). HDACs also facilitate MYC's oncogenic function as a transcription factor, as they are often recruited to the promoter regions of some MYC-targeted genes to facilitate MYC-mediated regulation of transcription. HDAC inhibitors thus decrease MYC gene expression and restore the expression of genes coordinately suppressed by MYC family members and HDACs in multiple cancer cell types, including DLBCL and NMC (19–25).

Blockade of the PI3K pathway has also been shown to suppress MYC activity by inhibiting MYC gene transcription (26) and...
decreasing MYC protein stability. Activation of PI3K signaling either through mutational activation of PI3K or through loss of the tumor suppressor PTEN leads to activation of the effector kinase AKT, which in turns inhibits GSK3β through phosphorylation (27). Active GSK3β normally phosphorylates MYC at the threonine 58 (Thr58) residue, which facilitates the ubiquitin–proteasome degradation of MYC protein. PI3K inhibition thus decreases MYC protein stability by releasing GSK3β from AKT-mediated inactivation and therefore promoting MYC Thr58 phosphorylation and subsequent degradation (28–31). Indeed, pharmacologic inhibition of the PI3K pathway is able to induce cell death in PTEN-deficient DLBCL cells as a result of MYC down-regulation (32). PI3K inhibition also potentiates MYC down-regulation and cell death in MYC-dependent NMC cells (33).

Synergistic antitumor effect between HDAC and PI3K inhibition has been reported in DLBCL xenograft tumors (34) and in MYC-driven mouse medulloblastoma cells in vitro (16). However, the impact of this combination approach on MYC regulation has not been demonstrated. Because HDAC and PI3K pathways are both involved in MYC regulation, we hypothesized that simultaneously targeting these two pathways may be an effective way to suppress MYC.

CUDC-907 is an orally bioavailable small-molecule dual HDAC and PI3K inhibitor targeting class I and II HDACs and the PI3Kα, β, and δ isoforms. Its broad antitumor activities in hematologic and solid tumors have been previously reported by us (35). In a recent phase I study to evaluate the safety, tolerability, and preliminary activity of CUDC-907 in patients with relapsed or refractory lymphoma or multiple myeloma, and preliminary activity of CUDC-907 in patients with relapsed or refractory lymphoma or multiple myeloma, we demonstrated that MYC-dependent cancer models would be responsive to simultaneous HDAC and PI3K inhibition with CUDC-907. In this study, we demonstrated that the combination of HDAC and PI3K inhibition synergistically decreases MYC protein levels and induces apoptosis in DH DLBCL cells. Moreover, CUDC-907 potently suppresses MYC at least partially through decreasing MYC gene transcription and reducing MYC protein stability. We also demonstrate here the antitumor activity of CUDC-907 in vitro in DLBCL and NMC cells, and in vivo in MYC-altered DLBCL and MYC-dependent NMC xenograft models. MYC transgenic tumor syngenic models, and MYC-amplified solid tumor PDX models. Our results provide further evidence that CUDC-907 may have broad utility in cancers that are driven by MYC upregulation.

Materials and Methods

Reagents

CUDC-907 was synthesized in-house as described previously (35). Panobinostat (LBH-589), pemetrexed (GDC-0941), 1-BET-762, 1Q1, OTX015, pan caspase inhibitor Z-VAD-FMK and proteasome inhibitor MG-132 were purchased from Selleck Chemicals. For in vitro assays, pan-caspase inhibitor Z-VAD-FMK and proteasome inhibitor MG-132 were dissolved in dimethyl sulfoxide (DMSO) to generate 50 mmol/L stock solutions. Other compounds were dissolved in DMSO to generate 1,000× stock solutions and stored at −80°C for single use only. For in vitro studies, CUDC-907 was formulated in 30% Captisol (Ligand Pharmaceuticals, Inc).

Cell culture

NMC cell lines were in-licensed from Dr. Christopher French (Harvard Medical School, Boston, MA). No authentication was done by the authors. Other cancer cell lines were purchased from the ATCC (Manassas, VA) and German Collection of Microorganisms and Cell Cultures within one year of the study. No authentication was done by the authors. NMC cells were maintained in RPMI1640 + GlutaMAX (Gibco by Life Technologies) medium supplemented with 10% FBS (Gibco) and incubated at 37°C in a humidified atmosphere of 5% CO2. Other cell lines were maintained as recommended by the source. Growth media were changed every 2 to 3 days and cells were maintained at a density of 2 × 104 to 4 × 105 cells/mL. Cells at exponential growth stage were used for all experiments described below.

Cancer cell proliferation and caspase induction assays

Cells were seeded at densities of 5 × 103 per well for proliferation assay or 2 × 104 per well for caspase assay in 96-well flat-bottomed plates with the recommended culture medium. Cells were then incubated with indicated compounds at various concentrations for indicated amount of time in recommended growth medium supplemented with 10% (v/v) FBS. Cell viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Caspase-3/7 induction was assessed using the Caspase-Glo 3/7 Assay (Promega). GraphPad Prism 5.0 (GraphPad Software) was used for curve fitting and IC50 calculation. Two independent experiments with duplicates were performed for each experiment.

Quantitative PCR experiments

Cells were seeded at densities of 3 × 104 per mL in 24-well plates with the recommended culture medium and incubated with indicated compounds at various concentrations for indicated amount of time. Cells were then pelleted by centrifugation at 10,000 × g at 4°C for 10 minutes, and immediately lysed in buffer RLT Plus (Qiagen). RNA was purified from lysed cells using RNeasy columns (Qiagen) following the manufacturer’s instructions. cDNA was generated with SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen by Life Technologies) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using TaqMan Real-Time PCR Master Mix and TaqMan Gene Expression Assay for MYC (Hs00153408_m1) and PPIA (Hs04194521_s1) on an Applied Biosystems qPCR instrument (Applied Biosystems by Life Technologies). Relative expression was calculated with the comparative Ct method using housekeeping gene PPIA as loading control.

Western blot analysis and immunocytochemistry

Snap-frozen tumor samples were pulverized with a mortar and pestle on dry ice and transferred to tubes containing cold lysis buffer (Halt phosphatase inhibitors (Thermo Scientific). Complete mini protease cocktail tablets (Roche Diagnostics), and T-PER Tissue Protein Extraction Reagent (Thermo Scientific)). One cold stainless steel bead (BioSpec Products) was placed into each tube and the samples were then lysed using a Qiagen TissueLyser (Qiagen) in cold blocks. The samples were then centrifuged twice at 16,000 × g at 4°C for 15 minutes. The samples were diluted with...
Cells were seeded at densities of $3 \times 10^5$ per mL in 24-well plates with the recommended culture medium and incubated with indicated compounds at various concentrations for an indicated amount of time. Cells were then collected and centrifuged at 10,000 $\times$ g at 4°C for 10 minutes. Cell pellets were washed with cold 1× PBS and lysed and sheared in 1× SDS sample buffer (Sigma-Aldrich). Equal amounts of lysates from each sample were loaded per lane onto NuPage gels (Invitrogen). Following electrophoresis, samples were transferred to a nitrocellulose membrane (Invitrogen). Membrane blocking and incubation with antibodies was performed using standard procedures. Immunoblots were probed with the antibody of interest: anti-phospho-AKT (Ser473), anti-AKT(pan), anti-phospho-GSK-3β (Ser9), anti-GSK-3β, anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), anti-p44/42 MAPK (ERK1/2), anti-BCL2, anti-MCL1, anti-c-MYC, anti-phospho-c-MYC (Ser62), anti-cleaved caspase-3 (Asp175), anti-Tubulin (Cell Signaling Technology); anti-phospho-c-MYC (Thr58; Thermo Scientific); anti-acetyl-Histone H3 (Millipore). Membranes were cut based on the size of markers and probed separately. Tubulin was used as loading control on each membrane, and markers from the same membranes were boxed together in figures. IRDye 680- or 800CW-conjugated secondary antibodies (LI-COR Biosciences) were used for detection. The membranes were scanned and the signal intensities were quantified on a LI-COR Odyssey Infrared Imaging System. The intensities of each marker were normalized by the intensity of tubulin from the sample membrane. Ratios of phosphorylated and total proteins were calculated according to the formula below using MYC as an example, where pMYC represents intensity of phosphor-MYC; Tubulin$_{\text{MYC}}$ represents the intensity of tubulin from the same membrane as pMYC; MYC represents intensity of total MYC; and Tubulin$_{\text{MYC}}$ represents the intensity of tubulin from the same membrane as the total MYC. 

**Ratio of MYC phosphorylation** $= (\text{pMYC} / \text{Tubulin}_{\text{MYC}}) / (\text{MYC}/\text{Tubulin}_{\text{MYC}})$

For dot Western blot analyses, a vacuum-based dot blot apparatus was used (Whatman Minifold I 96-well slot-blot array system, Sigma-Aldrich). The lysates were spotted through circular templates directly onto the membrane. Membrane blocking and incubation with antibodies were performed using standard protocols as described above for the Western blot assay. Tubulin was used as the loading control. MYC protein levels were calculated by the intensity of MYC normalized by the intensity of tubulin from the same dot on the same membrane.

**Combination synergy calculation**

The combination synergy between panobinostat and pictilisib in MYC protein regulation was determined using two mathematic methods, Chou–Talalay equation and Bliss independent model. The MYC protein levels were calculated by the intensity of MYC normalized by the intensity of tubulin from the same dot blot on the same membrane as described above. Serial dilutions of panobinostat or pictilisib alone, or their combination, were tested in a matrix format to get different constant molar ratios: 303:1, 100:1, 33:1, 11:1, 3.7:1, 1.2:1, or 0.41:1 of pictilisib and panobinostat. The combination index (CI) of each combination was calculated by the Chou–Talalay equation as described elsewhere (37) using Compusyn software (38) (CombioSyn, Inc.).

For MYC protein level decrease, the Bliss independence model is defined by the equation $E(d_1,d_2) = E(d_1) + E(d_2) - E(d_1)E(d_2)$, where $E(d_1,d_2)$ is the additive effect of panobinostat and pictilisib as predicted by their individual effects $E(d_1)$ and $E(d_2)$. For caspase-3/7 induction, the Bliss independence model is defined by the equation $E(d_1,d_2) = E(d_1) + E(d_2)$. 

**Differential gene expression analysis**

Cells were pelleted by centrifugation at 10,000 $\times$ g at 4°C for 10 minutes, and immediately lysed in buffer RLT Plus (Qiagen). RNA was purified from lysed cells using RNeasy columns (Qiagen) following the manufacturer’s instructions. Total RNA input was used to construct RNASeq libraries using the TruSeq RNA Sample Preparation Kit (Illumina) following the manufacturer’s instructions. Sequencing was performed on a HiSeq 2500 System (Illumina). The bioconductor package Limma was used for analysis of RNA-seq data (39, 40). RNA-seq counts for 10 conditions (control + 2 doses + 2 timepoints for 2 cell lines) were preprocessed using the voom function (41), and linear models fit using the lmFit function. Specifically, a full model was fit (expression ~ 0 + Treatment+ group + cellline+ group + dose.group + timepoint) and the coefficients extracted for the Treatment+group effect.

Two MYC target gene sets from the MSigDB Hallmark gene set "HALLMARK_MYC_TARGETS_V1" and "HALLMARK_MYC_-_TARGETS_V2" were used for gene set enrichment analysis (42). Clustering of genes was performed using complete linkage with Euclidean distance as a similarity metric. Correlations were computed using Spearman rank correlation test. The RNA-seq data are deposited at GEO website, accession number GSE89339.

**Lentivirus-mediated overexpression of MYC in WSU DLCL2 cells**

Cells were plated in 6-well tissue culture plate (Becton Dickinson and Company) at a density of $7 \times 10^5$ cells per well and incubated at 37°C with 5% CO$_2$ for 20–24 hours prior to lentivirus infection. Cells were then infected with CMV-C-MYC lentivirus and vector control (Cellomics Technology) in polybrene-containing medium (6 μg/mL, Sigma-Aldrich) and incubated at 37°C with 5% CO$_2$. After overnight incubation, the CMV-C-MYC lentivirus-containing medium and vector control-containing medium were removed and replaced by fresh complete growth medium. Cells continued to be incubated at 37°C with 5% CO$_2$.

**In vivo tumor models and efficacy studies**

E14-Myc mice (B6.Cg-Tg[IfgyMyc]22Bnn/J) were obtained from The Jackson Laboratory. The E14-Myc transgenics were monitored weekly to identify any mice with malignant disease. Mice were evaluated for any visible or palpable lumps, a hunched posture, tachypnea, a swollen belly, or ruffles in the fur and sacrificed promptly upon the appearance of any such symptoms. Lymphomas that emerged in lymph nodes were carefully dissected from sacrificed mice, washed in PBS, cut into <3-mm pieces, and implanted into C57BL/6J female mice (The Jackson Laboratory).

Six- to 9-week-old female immunodeficient athymic (nude nu/nu CD1-), Fox Chase SCID Beige, BALB/c nude mice, or...
Figure 1. HDAC and PI3K inhibition synergistically downregulates MYC and induces caspase-3/7 activation in MYC-altered DLBCL cells. A, Dot Western blot analysis of MYC protein (left) and tubulin (right) in WSU DLCL2 cells after 24 hours of treatment with various concentrations of HDAC inhibitor panobinostat, PI3K inhibitor pictilisib, or their combination at different concentrations as indicated. MYC and tubulin signals were captured from the same membrane at different fluorescence wavelength. B, Intensities of MYC signals are normalized by tubulin quantified from the dot Western blot analysis shown in A, and normalized to DMSO-treated cells. C, Combination indexes (CI) were calculated from the data presented in B using CompuSyn software. (Continued on the following page.)
immunocompetent C57Bl/6 mice obtained from Charles River Laboratories were either subcutaneously injected with 2 x 10^6 to 3 x 10^6 cells in a medium suspension of 100 to 200 μL (WSU DLCL2 and 10-15 NMC models) or inoculated with tumor fragments (2- to 3-mm in diameter) collected from stock mice growing primary human cancer tissues (ES2263, U10377, and CR2506 models). U2932 tumors, or genetically engineered Eμ-Myc tumors (F2-3 and M2-5) into the right hind flank region. The treatment was started when the average tumor size reached approximately 100 to 200 mm^3 (WSU DLCL2, U2932, 10-15, ES2263, U10377 and CR2506 models) or one day after inoculation (F2-3 and M2-5 Eμ-Myc models). Varying doses of CUDC-907 or vehicle (30% Capsisol) were administered orally as per Institutional Animal Care and Use Committee guidelines. Tumor size was measured twice weekly and the volume was expressed in mm^3 using the formula, V = 0.5a x b^2, where a and b were the long and short diameters of the tumor, respectively. The tumor size was then used for the calculation of tumor growth inhibition (TGI) = [1 - (T1 - T0)/(C1 - C0)] x 100, where C1 = mean tumor volume of control mice at time t; T1 = mean tumor volume of treated mice at time t; C0 = mean tumor volume of control mice at time 0; and T0 = mean tumor volume of treated mice at time 0.

**Statistical analysis**

Differences between values obtained in tumors treated with different experimental conditions were determined using the Student t test on GraphPad Prism 5.0 (GraphPad Software). P < 0.05 was considered statistically significant.

**Results**

**HDAC and PI3K inhibition synergistically decreases MYC protein levels and induces apoptosis**

Because HDAC and PI3K pathways both regulate MYC levels, we evaluated the potential synergy between HDAC inhibitor panobinostat (LBH-589) and PI3K inhibitor pictilisib (GDC-0941) in reducing MYC protein levels. In WSU DLCL2 DH DLBCL cells, synergistic suppression of MYC protein levels was observed when panobinostat was combined with pictilisib at multiple concentrations with the best combination index (CI) at 0.38, indicating synergism, when evaluated with CompuSyn software (Fig. 1A–C). Synergy was also confirmed using the Bliss independence model, showing that the combined effect is greater than that predicted by their individual potencies (Fig. 1D). Synergy between panobinostat and pictilisib in caspase-3/7 induction was also observed in the same cell line using the Bliss independence method (Fig. 1E). These results suggested that HDAC and PI3K pathway inhibition synergistically reduces MYC protein levels and induces apoptosis in MYC-driven DLBCL cells, providing a rationale for testing CUDC-907 in MYC-altered cancers.

**CUDC-907 suppresses MYC protein, inhibits cell growth, and induces apoptosis in MYC-altered DLBCL cell lines**

On the basis of the synergy observed between HDAC and PI3K inhibition in MYC regulation, we next evaluated the antitumor activity of the dual function HDAC and PI3K inhibitor CUDC-907 (chemical structure shown in Fig. 2A) in WSU DLCL2 cells, and observed that CUDC-907 inhibits cell growth and induces apoptosis with nanomolar potency (Fig. 2B). The antitumor activity of CUDC-907 is more potent than the HDAC inhibitor panobinostat and the PI3K inhibitor pictilisib in a panel of DLBCL cell lines tested (Fig. 2C and D), including cell lines with MYC alternation such as translocation, amplification, or overexpression, and cell lines that harbor both MYC and BCL2 translocations (DH), or MYC overexpression and BCL2 amplification (“double-expressor”), which have a particularly poor prognosis (43). The antitumor activity of CUDC-907 appeared to be independent of DLBCL cell-of-origin subtypes as both activated B cell-like (ABC) and germinal center B cell (GCB) type DLBCL cells were sensitive to CUDC-907. Consistent with its potent effect in inhibiting cell growth and inducing apoptosis, CUDC-907 is also more effective in downregulating MYC proteins in WSU DLCL2 cells, when compared with pictilisib and panobinostat (Fig. 2E).

To understand the mechanism underlying the antitumor activity of CUDC-907 in DH and “double-expressor” DLBCL cells, we evaluated the changes induced by CUDC-907 in protein markers related to the HDAC, PI3K, and MYC pathways, including BCL2, a known HDAC target gene (44) that cooperates with MYC to induce lymphomagenesis (45), and MCL1, which is transcriptionally regulated by MYC (46) and whose downregulation has been shown to contribute to synergy between HDAC and PI3K inhibitors in DLBCL cells (47). As shown in Fig. 2F, 6 hours of CUDC-907 treatment at nanomolar concentrations led to a dose-dependent accumulation of H3 acetylation (Ac-H3) and reduction of PI3K pathway markers, such as phosphorylated AKT (pAKT) and phosphor-GSK3β, in WSU DLCL2 cells, indicating potent on-target inhibition of HDAC and PI3K activities, respectively. HDAC or PI3K inhibition is also induced by panobinostat or pictilisib, respectively (Supplementary Fig. S1A–S1B). Dose-dependent reduction of MYC protein levels was observed 6 hours post-CUDC-907 treatment (Fig. 2F). At the same time point, the levels of MYC phosphorylation at the Ser62 site, which stabilizes the MYC protein, decrease together with total MYC protein levels (Fig. 2F and G). However, no decrease was observed at the Thr58 phosphorylation site, which targets MYC for ubiquitin–proteasome degradation, even though the total MYC protein levels decreased dramatically (Fig. 2F and G). In comparison, the levels of BCL2 and MCL1 decrease after 24 hours of treatment, but not 6 hours post-treatment (Fig. 2F). The levels of BIM, a proapoptotic BH3-only protein that is a negative regulator of BCL2, did not decrease after 6 or 24 hours of treatment (Fig. 2F). Similar results were also observed in the “double-expressor” cell line U2932.
harboring BCL2 amplification and MYC overexpression (Supplementary Fig. S1C and S1D). These results suggest that MYC reduction is an early event induced by CUDC-907 in MYC-altered cell lines. Interestingly, CUDC-907 shifted the balance between the two phosphorylation sites on MYC, Thr58 which promotes MYC protein degradation and Ser62 which stabilizes MYC protein.

CUDC-907 decreases MYC gene transcription, induces MYC protein degradation, and suppresses MYC function

To better understand the MYC regulation mechanism of CUDC-907, we next evaluated the kinetics of MYC Thr58 phosphorylation and MYC protein degradation in WSU DLCL2 cells treated with nanomolar concentrations of CUDC-907 for varying lengths of time ranging from 15 minutes to 6 hours. The MYC protein levels start to decrease 2 hours post-CUDC-907 treatment, and continue decreasing until the last time point tested in this experiment, 6 hours (Fig. 3A). The accumulation of MYC Thr58 phosphorylation, reflected by the ratios of phospho-MYC (Thr58) to total MYC, also started after 2 hours of CUDC-907 treatment, and this ratio increased about 12 folds after 6 hours of treatment with 1 μmol/L CUDC-907 (Fig. 3B). The reduction in MYC protein levels induced by 6 or 24 hours of CUDC-907 treatment is not attenuated by caspase inhibitor Z-VAD-FMK (Fig. 3C), which inhibits the caspase activation induced by CUDC-907 in these cells (Fig. 3C, Supplementary Fig. S2A and B). In fact, no caspase activity was induced by 2 hours of CUDC-907 treatment in these cells (Supplementary Fig. S2A). These results indicate that accumulation of MYC Thr58 phosphorylation and caspase-independent degradation of MYC protein are early events induced by CUDC-907.

Because both HDACs and PI3K are involved in the transcription of MYC, we next evaluated changes in MYC mRNA levels induced by CUDC-907 treatment. As shown in Fig. 3D, CUDC-907 potently and dose dependently decreased MYC mRNA levels at nanomolar concentrations after 6 hours of treatment. HDAC inhibitor panobinostat and PI3K inhibitor pictilisib also decrease MYC mRNA levels, but are less potent than CUDC-907.

To further dissect the role of CUDC-907 in MYC regulation, MYC gene was expressed under the control of the CMV promoter and the CMV-driven MYC expression partially rescued the caspase activation (Fig. 3F) and increased 0.5-fold as a result of the CMV-driven MYC expression bypassing regulations on the c-MYC promoter. MYC protein level increased 0.5-fold as a result of the CMV-driven MYC expression mediated by lentivirus transduction in WSU DLCL2 cells to investigate the potential involvement of proteasome in this early reduction in MYC protein levels induced by CUDC-907. As shown in Fig. 3H, pharmacologic inhibition of the proteasome function by proteasome inhibitor MG-132 partially blocks the early reduction in MYC protein levels induced by CUDC-907 treatment in WSU DLCL2 cells, suggesting that proteasomal degradation of MYC proteins is involved in this process.

Collectively, our results indicate that both the transcriptional regulation of MYC gene expression and the proteasome-mediated MYC protein degradation contribute to the MYC suppression induced by CUDC-907.

RNA-seq analysis was conducted to evaluate the effect of CUDC-907 on MYC-associated genes. When WSU DLCL2 and DOHH2 DH DLBCL cells were treated with CUDC-907 at nanomolar concentrations for 6 or 12 hours, a total of 948 differentially regulated genes were identified (Supplementary Fig. S3A). We first looked for enrichment of regulated MYC target genes within the "HALLMARK_MYC_TARGETS_V2" gene set in the Molecular Signatures Database (MSigDB). 16 and 8 genes, respectively, are represented in the CUDC-907–regulated 948-gene set (Supplementary Fig. S3B). This corresponds to a 12.5-fold ($P_{adj} = 1.67e^{-05}$) and 7.2-fold ($P_{adj} = 5.59e^{-06}$) enrichment of MYC target genes in the CUDC-907–regulated gene set, respectively.

Second, we investigated whether CUDC-907 is causing expression changes of genes whose mRNA levels are associated with MYC protein levels in DLBCL patients. We computed the correlation coefficients ($r$) between gene expression levels and MYC protein levels across all samples in the TCGA DLBCL dataset. For genes in each bin defined by their correlation with MYC protein levels in DLBCL patients, we calculated the percentage of genes whose mRNA levels are upregulated (logFC>$0$) or downregulated ($logFC<0$).
(logFC < 0) after CUDC-907 treatment (Fig. 3I). Genes with a positive correlation to MYC protein levels (r > 0.3) are more frequently (~70%) downregulated after CUDC-907 treatment, whereas genes with a negative correlation to MYC protein levels (r < −0.3) are more frequently (~65%) upregulated after CUDC-907 treatment. This result indicates that the mRNA expression changes induced by CUDC-907 in the two DLBCL cell lines are negatively correlated with correlation coefficients between expression levels and MYC protein level in the TCGA DLBCL dataset (r = −0.23; P = 5.20e−218; Supplementary Fig. S3C), suggesting that CUDC-907 changes the mRNA expression of a subset of MYC-associated genes in DLBCL. Together, these results indicate that CUDC-907 is able to downregulate MYC by inhibiting MYC transcription and reducing MYC protein stability, and it may also be able to at least partially reverse MYC-dependent transcriptional regulation of a subset of MYC-associated genes in DLBCL.

Antitumor activity of CUDC-907 in MYC-driven DLBCL xenograft models and Eμ-Myc transgenic tumor syngeneic models of B-cell lymphoma

To determine whether CUDC-907 has activity against MYC-driven B-cell lymphomas in vivo, we evaluated the effect of CUDC-907 in severe combined immunodeficient beige (SCID-beige) mice bearing established WSU DLCL2 or U2932 xenograft tumors. When orally administered at 100 mg/kg once daily every day or 5 days on and 2 days off, CUDC-907 led to significant growth suppression of the WSU DLCL2 DH GC B-CLL xenografts and achieved near stabilization in the “double expressor” U2932 ABC DLCL xenograft tumors, with tumor growth inhibition (TGI) of 69% and 97%, respectively, at the end of the efficacy study (Fig. 4A and B) without causing severe body weight loss or noticeable toxicity (Supplementary Fig. S4A-B).

To further provide confirmation that CUDC-907 has antitumor activity against MYC-dependent B-cell lymphomas, we treated C57BL/6 mice bearing Eμ-Myc transgenic tumors of B-cell lymphoma (48) with CUDC-907 and found that CUDC-907 markedly reduced tumor growth with a TGI of 72% and 58% in two models derived from tumors that spontaneously arose in two Eμ-Myc transgenic mice (Fig. 4C and D) without inducing significant body weight loss or obvious toxicity (Supplementary Fig. S4C-D). In addition, pharmacodynamic analysis of WSU DLCL2 xenograft tumors treated with 5 doses of CUDC-907 at 100 mg/kg showed a dramatic reduction of MYC protein levels (~90%) and MYC downregulation was also observed in other models, such as the Daudi Burkitt lymphoma model harboring MYC translocation (Supplementary Fig. S5), in which we previously reported efficacy of CUDC-907 (35). These results demonstrate the antitumor efficacy of CUDC-907 and provide evidence of CUDC-907–induced MYC downregulation in B-cell lymphomas in vivo.

Antitumor activity of CUDC-907 in MYC-amplified solid tumor PDX models

To determine the effect of CUDC-907 in other MYC-driven tumor models, CUDC-907 was evaluated in three PDX models harboring MYC gene amplifications, including an esophageal cancer with 12 copies of the MYC gene (ES2263), a non–small cell lung cancer with 7 copies of the MYC gene (LJI0377), and a colorectal cancer with 6 copies of the MYC gene (CR2506). Suppression of tumor growth was observed in response to treatment with CUDC-907 at 100 mg/kg 5-days-on and 2-days-off for 21 days in all three models tested, with TGI of 71%, 54%, and 54% in ES2263, LJI0377, and CR2506, respectively (Fig. 5A–C), without causing significant body weight loss (data not shown). These results demonstrate the broad antitumor activity of CUDC-907 in MYC-amplified solid tumors.

CUDC-907 inhibits NMC growth in vitro and in vivo

To explore the antitumor activity of CUDC-907 in MYC-dependent solid tumors without genetic alterations directly affecting the MYC gene, we next evaluated the efficacy of CUDC-907 in NMC cells harboring BRD–NUT fusions. We found that CUDC-907 is more potent in inhibiting the growth of NMC cells in vitro than BET bromodomain inhibitors (e.g., JQ1, I-BET-762, and OTX015) that directly target the BRD–NUT fusion protein and block growth of NMC cells by inducing MYC downregulation (6, 49; Fig. 6A).

Similar results were also observed in two MYC-driven DLBCL cell lines, WSU DLCL2 and U2932, when treated with CUDC-907 and BET inhibitors (data not shown). In addition, CUDC-907 also more potently reduces the levels of MYC proteins (Fig. 6B) than either panobinostat or pictilisib in MYC-driven NMC cell lines.
Figure 4.
Antitumor activity of CUDC-907 in human DLBCL cell line–derived xenograft models and the Eμ-Myc transgenic tumor mouse syngeneic models of B-cell lymphoma. A and B, Growth curves of established WSU DLCL2 (A) and U2932 (B) DLBCL xenograft tumors engrafted in severe combined immunodeficient beige (SCID-beige) mice upon oral administration of CUDC-907. Each graph describes the tumor volume (y-axis) as a function of the time after the initiation of treatment (x-axis). Each datapoint represents the mean volume of 6 (A) or 9 (B) independent tumors. Error bars represent SEM. WSU DLCL2 xenografts were treated with vehicle control (30% captisol) or CUDC-907 once a day for 21 days at 100 mg per kg body weight (mg/kg) as indicated. U2932 xenografts were treated with vehicle control (30% captisol) or CUDC-907 daily in a 5-days-on and 2-days-off schedule for 20 days at 100 mg/kg body weight as indicated. P values are as indicated.

C and D, Growth curves of xenograft tumors derived from two independent B-cell lymphoma tumors spontaneously occurring in Eμ-Myc transgenic mice and engrafted in C57BL/6 mice upon oral administration of CUDC-907. Each graph describes the tumor volume (y-axis) as a function of the time after the initiation of treatment (x-axis). Each datapoint represents the mean of 10 (C) or 9 (D) independent tumors. Error bars represent SEM. All mice were treated with vehicle control (30% captisol) or CUDC-907 daily at a 5-days-on, 2-days-off schedule for 12 (C) or 13 (D) days at 100 mg/kg body weight as indicated. P values are as indicated.

E, Immunoblot analysis of MYC and tubulin was performed on lysates from WSU DLCL2 xenograft tumors treated with 5 oral daily administrations of vehicle control (30% captisol) or CUDC-907 at 100 mg/kg body weight as indicated. Three independent tumors at 1, 6, 12, and 24 hours after the fifth administration as indicated. Individual blots are boxed together. Tubulin was used as a loading control.

*P < 0.05; **P < 0.01; ***P < 0.001.
These results suggest that CUDC-907 is able to inhibit the growth of MYC-dependent NMC cells and reduce MYC protein levels.

In vivo, significant tumor growth suppression (TGI = 94%; Fig. 6C) and improved survival (Fig. 6D) were observed when BRD4–NUT fusion-positive NMC xenograft tumors were treated with 100 mg/kg of CUDC-907 on a 5-days-on and 2-days-off schedule for 24 days or 63 days, respectively, without inducing significant body weight loss or obvious toxicity (Supplementary Fig. S7).

These results indicate that CUDC-907 is effective in MYC-dependent NMCs.

Taken together, our results indicate that CUDC-907 effectively downregulates MYC protein levels in MYC-altered and MYC-dependent cells and tumor models, which at least is partially attributable to the synergy between simultaneous HDAC and PI3K inhibition in MYC regulation. These results provide a strong rationale for the clinical development of CUDC-907 in MYC-driven cancer indications.

Figure 5.
CUDC-907 inhibits tumor growth in MYC-amplified patient-derived xenograft (PDX) models. Growth curves of established PDX tumors derived from (A) esophageal carcinoma (ES2263), (B) non–small cell lung carcinoma (LU0377), and (C) colorectal cancer (CR2506) engrafted in immunodeficient BALB/c nude mice upon oral administration of CUDC-907. Each graph describes the tumor volume (y-axis) as a function of the time after the initiation of treatment (x-axis). Each datapoint represents the mean tumor volume of eight (A and B) or four (C) independent tumors. Error bars represent SEM. The mice from all three PDX models were treated with vehicle control (30% captisol) or CUDC-907 daily with a 5-days-on, 2-days-off schedule for 21 days at 100 mg/kg body weight as indicated. P values are as indicated: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 6.
Antitumor activity of CUDC-907 against MYC-dependent NUT midline carcinoma (NMC) cells in vitro and in vivo. A, Growth inhibition IC50 values of CUDC-907 and three BET inhibitors (I-BET-762, JQ1, and OTX015) in three BRD–NUT fusion-positive NMC cell lines. Cell viability after 72-hour incubation was assessed by the CellTiter-Glo assay. Growth inhibition IC50 values for each compound were determined by GraphPad Prism 5. (Continued on the following page.)
Discussion

MYC-driven tumors, such as DLBCL, NMC, and other MYC-amplified solid tumors, represent a significant unmet medical need due to their poor prognosis, but efforts to directly target MYC have not been successful to date. Here we show for the first time that simultaneous inhibition of HDACs and PI3K, upstream regulators of MYC gene expression and protein stability (Fig. 6E), synergistically downregulates MYC protein levels. This observation provided the foundation for testing the dual HDAC and PI3K inhibitor CUDC-907 in MYC-driven tumor models. Indeed, our results showed that CUDC-907 potently reduce MYC protein levels in MYC-dependent cancers regardless of whether MYC is genetically altered via translocation or amplification as in DLBCL and some solid tumor types, or epigenetically upregulated, as is the case in "double-expressor" DLBCL and NMC. This pharmacodynamic effect was also confirmed in tumor tissues from animal models, indicating that a sufficient therapeutic window exists in vivo. The broad antitumor activities of CUDC-907 in multiple in vivo models representing several indications suggest that it may be an effective therapy across a wide range of hematologic and solid tumor types that are known to be dependent on MYC. Because of the homology between MYC family proteins and potential similarities in their regulation, it will be interesting to determine in future studies whether CUDC-907 also downregulates the MYC homologs MYCN and MYCL and whether CUDC-907 has activity in tumors where alterations in these genes are driving events. Given the pleiotropic effects of HDAC inhibition and the multiple downstream effectors of PI3K, however, it is likely that the effects of CUDC-907 are not completely attributable to MYC suppression and that CUDC-907 will have activity in tumors caused by other genetic or epigenetic alterations. Indeed, CUDC-907 did suppress tumor growth of Karpas-422 xenografts that do not express high levels of MYC, albeit not to the same extent as MYC-overexpressing models (Supplementary Fig. S8).

Additional studies are also needed to further dissect out the primary and secondary effects of CUDC-907 to fully understand its broad impact on oncogenic networks. For example, we detected relative accumulation of MYC Thr58 phosphorylation and caspase-independent reduction of MYC protein as early as 2 hours after CUDC-907 treatment in both wild-type WSU DLCL2 cells and the ones express MYC under the control of CMV promoter, suggesting that this early reduction of MYC protein might be a primary result of the drug’s effect on the PI3K pathway involving no transcriptional regulation. In comparison, levels of BCL2 and MCL1 remained unchanged until 6 to 24 hours post-treatment (Fig. 2F) when apoptosis starts (Supplementary Fig. S2A), suggesting that these may be secondary or subsequent downstream effects of CUDC-907 treatment. One may suspect that the decrease of BCL2 and MCL1 levels might be part of the massive protein degradation that typically occurs during the late stage of apoptosis. However, it appears not to be the case for CUDC-907 in this setting, as the levels of the proapoptotic protein BIM kept increasing throughout the 24-hour treatment, indicating specific drug effects instead of massive protein degradation. Thorough studies focusing on the mechanism of CUDC-907’s antitumor actions may help to identify other sensitive tumor types in addition to the MYC-driven cancers.

Regardless of the total contribution of MYC suppression to the antitumor activity of CUDC-907, our preclinical findings showing that CUDC-907 effectively suppresses MYC and has activity in multiple models of MYC-driven cancers suggests that patients whose tumors harbor genetic alterations in MYC or are dependent on MYC overexpression may benefit from CUDC-907 treatment. This is consistent with the results of the recently published phase 1 study of CUDC-907 in patients with relapsed or refractory lymphoma or multiple myeloma. In the interim analysis of this trial, the single-agent activity of CUDC-907 was observed in patients with DLBCL and transformed FL, which is commonly driven by MYC overexpression, amplification, or translocation, and led to two complete and three partial responses (36). To our knowledge, single-target HDAC or PI3K inhibitors have not shown such promising efficacy in this population. Moreover, CUDC-907 is generally well tolerated, causing manageable gastrointestinal and hematologic events that were similar to those observed with FDA-approved single-target HDAC and PI3K inhibitors. Of note, the combination of the MTDs of the FDA-approved HDAC inhibitors, vorinostat or panobinostat and the FDA-approved PI3K inhibitor idelalisib are not tolerable in animal models in our experience (33 and data not shown), suggesting that such combination strategies may not be feasible in patients. Although longer follow-up studies and additional randomized studies in larger cohorts are needed, the favorable safety profile and clinical efficacy of CUDC-907 suggests that this compound would have numerous advantages over combination treatment with single-target HDAC and PI3K inhibitors. Even though a dual-function single agent may lose the flexibility of independent dose adjusting, the clinical development process of this type of dual-function drug candidate is much simpler than combination therapy because the pharmacokinetics, and toxicity profile are more predictable and easier to manage.

Based on these preclinical findings, a phase 1 study to evaluate the safety, tolerability, and pharmacokinetics of CUDC-907 has been initiated in patients with advanced/refractory solid tumors, including NMC (NCT02307240), and given the encouraging clinical results in patients with DLBCL, a biomarker-driven
phase II trial (NCT02674750) has also been initiated to evaluate the activity of CUDC-907 with or without rituximab in patients with MYC-altered DLBCL in which the enrollment criteria include relapsed or refractory DLBCL patients who have MYC translocation, amplification, overexpression, or copy number gain. Future studies will explore other genetic and epigenetic backgrounds in which CUDC-907 might be effective as well as potential ways to utilize CUDC-907 in combination with other anitumor agents.

Disclosure of Potential Conflicts of Interest
A. Fattaey has ownership interest (including patents) in Curis, Inc. J. Wang is a director at Tesarollo. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K. Sun, A. Fattaey, J. Wang
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