Precision Targeting with EZH2 and HDAC Inhibitors in Epigenetically Dysregulated Lymphomas

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Abstract

Purpose: Both gain-of-function enhancer of zeste homolog 2 (EZH2) mutations and inactivating histone acetyltransferases mutations, such as CREBBP and EP300, have been implicated in the pathogenesis of germinal center (GC)-derived lymphomas. We hypothesized that direct inhibition of EZH2 and histone deacetyltransferase (HDAC) would be synergistic in GC-derived lymphomas.

Experimental Design: Lymphoma cell lines (n = 21) were exposed to GSK126, an EZH2 inhibitor, and romidepsin, a pan-HDAC inhibitor. Synergy was assessed by excess over bliss. Western blot, mass spectrometry, and coimmunoprecipitation were performed. A SU-DHL-10 xenograft model was utilized to validate in vitro findings. Pretreatment RNA-sequencing of cell lines was performed. MetaVIPER analysis was used to infer protein activity.

Results: Exposure to GSK126 and romidepsin demonstrated potent synergy in lymphoma cell lines with EZH2 dysregulation. Combination of romidepsin with other EZH2 inhibitors also demonstrated synergy suggesting a class effect of EZH2 inhibition with romidepsin. Dual inhibition of EZH2 and HDAC led to modulation of acetylation and methylation of H3K27. The synergistic effects of the combination were due to disruption of the PRC2 complex secondary to acetylation of RbAP 46/48. A common basal gene signature was shared among synergistic lymphoma cell lines and was characterized by upregulation in chromatin remodeling genes and transcriptional regulators. This finding was supported by metaVIPER analysis which also revealed that HDAC 1/2 and DNA methyltransferase were associated with EZH2 activation.

Conclusions: Inhibition of EZH2 and HDAC is synergistic and leads to the dissociation of PRC2 complex. Our findings support the clinical translation of the combination of EZH2 and HDAC inhibition in EZH2 dysregulated lymphomas.

Introduction

Enhancer of zeste homolog 2 (EZH2) is critical in the germinal center (GC) reaction and serves as the catalytic subunit of the Polycomb Repression Complex 2 (PRC2), inducing trimethylation of histone 3 lysine 27 (H3K27me3), a marker of transcriptional repression (1). During the GC reaction, the PRC2 complex recruits histone deacetyltransferase (HDAC) 1/2 and DNA methyltransferases (DNMT) to further inhibit transcription (2, 3).

Disturbances in epigenetic pathways have been implicated in lymphomagenesis. Aberrancy of histone methyltransferases, such as EZH2, has been associated with the development of GC-derived lymphomas, including diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (4, 5). Activating mutations in EZH2 have been implicated in 22% of GC-DLBCL and 7% to 12% of follicular lymphoma (4, 5). EZH2 dysregulation has been implicated in other lymphoma subtypes, including overexpression in some subtypes of T-cell lymphoma (TCL; refs. 6–9). Given the prevalence of EZH2 dysregulation in several malignancies, EZH2 inhibitors have been developed and demonstrate superior efficacy in mutated EZH2 GC-derived lymphoma cell lines compared with wild-type EZH2 cell lines (10–12). The preclinical activity of the EZH2 inhibitors in B-cell lymphomas has been replicated in the clinic by tazemetostat, a first-in-class EZH2 inhibitor, which demonstrated an overall response rate of 38%
Translational Relevance

Given the prevalence of enhancer of zeste homolog 2 (EZH2) mutations and histone acetyltransferase mutations in germinal center diffuse large B-cell lymphoma, the rational combination of EZH2 inhibition and histone deacetyltransferase (HDAC) inhibition was explored. Using a panel of 21 lymphoma cell lines, we demonstrate that exposure to dual inhibition of EZH2 and HDACs was synergistic in EZH2 dysregulated lymphomas. The synergistic effects of EZH2 and HDAC inhibition may be attributed to the disassembly of the PRC2 complex. In a mouse xenograft model of SU-DHL-10, the combination led to tumor growth delay and an improvement in overall survival. A basal common genetic signature among synergistic cell lines was identified using gene set enrichment analysis and metaVIPER analysis and correlates with therapeutic response. The novel combination of dual EZH2 and HDAC inhibition may serve as a future precision medicine therapeutic platform. A clinical trial to further explore this combination is in development.

Materials and Methods

Cell lines and culture

OCI-LY1, SU-DHL-2, SU-DHL-6, Pfeiffer, Farage, Toledo, Riva, HBL-1, Jeko-1, Z-138, H9, and H1 were obtained from the ATCC. OCI-LY7, OCI-LY10, SU-DHL-10, and OCI-LY3 were obtained from DSMZ. PF382 and P12 were gifts from the laboratory of Adolfo Fernando. TLOM-1 and MT-1 were obtained from Kyoto University; and MT-2 was obtained from Memorial Sloan Kettering Cancer Center. All cell lines were authenticated and screened for mycoplasma using the ATCC/Promega STR Authentication Testing Kit and Lonza MycoAlert, obtained between 2008 and 2016 and revised after 2 weeks. Experiments were performed between 2015 and 2018.

EZH2 PCR

Genomic DNAs from 21 lymphoma cell lines were extracted with cell culture DNA mini kit (Qiagen) and measured by NanoDrop 3300. PCR was performed by following the manufacturer’s instructions. Genomic DNA was amplified by PCR with AmpliTaq Gold DNA Polymerase, PE Buffer II, and MgCl2 (Applied Biosystems) using primers designed as follows: EZH2 Y641 forward, 5'-CAGGTCCTAGGATTATCAGTACGAT-3'; EZH2 Y641 reverse, GCAGAATCCAGCTGAAA-3'; EZH2 A677 forward 5'-GCCAAC-CTCTGAGACGTGA-3'; EZH2 A677 reverse 5'-CTGATCCATCATGAGACTGAAA-3'. PCR products were run on an agarose gel, purified using QIAquick PCR purification kit (28104), and sent for sequencing (Genewiz).

Cell viability assays

Cells were counted and resuspended based on their optimal density for log-phase growth. Cell viability assays were performed as previously described (17). Cells were exposed to romidepsin (Selleckchem). ACY957 (Acetlyon), GSK126 (Selleckchem), EPZ011989 (Epizyme), and CPI-1205 (Selleckchem). Synergy was assessed by excess over bliss (EOB; refs. 20, 21). Sensitivity to GSK126 and romidepsin as determined by mean IC_{50} was correlated with EZH2 mutation/overexpression and HAT mutations, respectively, using Prism GraphPad’s Student paired t test. Experiments were performed in triplicate and repeated at least twice.

Flow cytometry

Flow cytometry analysis was performed using FITC Annexin V Apoptosis Detection Kit with PI (Biolegend #640194) as previously described (16). Experiments were performed at least 3 times.

Coimmunoprecipitation

Immunoprecipitation was performed using the Pierce Co-Immunoprecipitation Kit (#26149). Columns were prepared
with 20 to 40 μg of antibody. Whole protein lysate was incubated with antibody. Flow through was collected, and column was washed and eluted. Antibodies used were anti-EZH2 (Cell Signaling Technology), anti-SUZ12 (Cell Signaling Technology), anti-RbAP 46/48 (Cell Signaling Technology), anti-EED (Millipore), anti-HDAC2 (Cell Signaling Technology), and anti-DNMT3L (Novus Biologicals). Experiments were performed at least 3 times.

Western blotting

Western blotting was performed as previously described (16). Antibodies used were as above. Experiments were performed at least 3 times.

Mass spectrometry for acetylation of PRC2 complex

Immunoprecipitation was performed using Thermo Scientific Pierce MS-Compatible Magnetic IP Kit. Protein was incubated with EZH2 or acetylated-lysine antibody. Antibody-bound proteins were eluted and run into SDS-PAGE. The excised gel lane pieces were reduced, alkylated, and digested in Trypsin Gold (Promega) digestion buffer (Thermo Fisher Scientific). Peptides were extracted with 70% acetonitrile (Thermo Fisher Scientific). The concentrated peptide mix was reconstituted in a solution of 2% ACN and 2% formic acid for MS analysis. Peptides were eluted from the column using a Dionex Ultimate 3000 Nano LC system. Using Thermo Fusion Tribrid mass spectrometer (Thermo Scientific), eluted peptides were electrosprayed. Mass spectrometer-scanning functions and high performance liquid chromatography (HPLC) gradients were controlled by the Xcalibur data system (Thermo Fisher). Experiments were performed at least twice.

Database search and interpretation of MS/MS data

Tandem mass spectra from raw files were searched against uniprot_human_170129.fasta database using the Proteome Discoverer 2.1 (Thermo Fisher). The mouse protein database was downloaded as FASTA-formatted sequences from Uniprot protein database (January 2017). The peptide mass search tolerance was 10 ppm with a required minimum sequence length of 7 amino acids. To calculate confidence levels and FDRs, Proteome Discoverer generates a decoy database and performs the search against this concatenated database (nondecoy + decoy). Scaffold (Proteome Software, Inc.) was used to visualize and filter to <1% FDR. Spectral counts were used for estimation of relative protein abundance.

HDAC short hairpin RNA

Human HDAC2 short hairpin RNA (shRNA) plasmids were purchased from Origene (#TG312495). HEK293 cells were plated in OPTI-MEM containing shRNA or scramble using Lipofectamine 3000 (Cat#L3000075). Cells were selected with puromycin, periodically analyzed by flow cytometry and fluorescent microscopy to monitor GFP levels until a stable cell line had been generated.

MS analysis and data handling for H3K27 acetylation and methylation

Histone extraction, derivatization, and tryptic digestion were adapted from previous works (22, 23). Peptides were resuspended in 0.1% TFA for LC-MS/MS analysis. Multiple reaction monitoring was performed on a triple quadrupole (QqQ) mass spectrometer (Thermo Fisher Scientific TSQ Quantiva) directly coupled with Ultimate 3000 Dionex nano-LC system. The following QqQ settings were used: collision gas pressure of 1.5 mTorr; Q1 peak width of 0.7 (FWHM); cycle time of 2 seconds; skimmer offset of 10 V; electrospray voltage of 2.5 kV. Modified and unmodified histone peptides monitored in the assay were selected based on previous reports (23). Raw MS files were imported and analyzed in Skyline software with Savitzky–Golay smoothing (24). Automatic peak assignments from Skyline were manually confirmed. Peptide peak areas from Skyline were used to determine the relative abundance of each histone modification. The relative abundances were determined based on the mean of three technical replicates with error bars representing the SD. Experiments were performed at least twice.

In vivo studies

Animals were maintained in accordance with an Institutional Animal Care and Use Committee–approved protocol (AC-AAAR9404). SU-DHL-10 (1 x 10⁶) was suspended in 50% Matrigel (BD Biosciences) and 50% PBS (Gibco) and subcutaneously injected into the flanks of 5- to 7-week-old beige/SCID female mice (Taconic Farms). Mice were randomly divided into 5 cohorts (n = 9–10) upon tumor volume reaching 80 to 100 mm³ as follows: (i) Normal saline: days 1, 4, 8, 15, and 18; (ii) GSK126: 100 mg/kg days 1, 4, 8, 11, 15, and 18; (iii) romidepsin: 2 mg/kg days 1, 8, and 15; (iv) GSK126 and romidepsin; (v) pretreatment with GSK126 (days 1, 4, 8, and 15), and followed by romidepsin on days 8, 15, and 22. Dosing was selected based on prior in vivo studies (11, 25, 26). Drugs were diluted in sterile normal saline and administered via i.p. route. Weight and tumor volume were evaluated 3x/week. Statistical analysis was performed using two-way ANOVA, and overall survival (OS) was estimated using the Kaplan–Meier method (GraphPad Software, Inc.)

Pharmacokinetic/pharmacodynamics in vivo studies

Plasma samples were collected at 0.25, 0.5, 2, 4, 8, and 24 hours after one-time infusion of GSK126 and romidepsin. Noncompartamental analysis was performed using Phoenix Winnonlin software (Certara) to define the maximum plasma concentration (Cmax), the time to maximum plasma concentration (Tmax), and the area under the plasma concentration time curve from t = 0 to the last data point (AUClast). Romidepsin and GSK126 were extracted by mixing 2:1 solution of serum/tissue homogenate in acetonitrile/methanol.

LC-MS/MS analysis was performed using Agilent 6140 triple quadrupole mass spectrometer (Agilent Technologies). Data acquisition and peak integration were done using MassHunter software v 3.1. The assay performance was validated for mouse serum samples according to FDA guidelines (27). Intra-assay precision and accuracy for romidepsin in mouse serum were 5.55% and 105.1%, respectively, whereas the interassay precision was 5.1%. For GSK126, the intra-assay accuracy was 99.35% with a precision of 1.55%, whereas the interassay precision was 2.83%.

RNA-SEQ

RNA was purified using the RNAeasy Plus Kit (QIAGEN). RNA concentration and integrity were verified using Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were generated using Illumina’s TrueSeq RNA sample Prep Kit v2, following the...
manifacter's protocol. Note that 2 × 75 bp paired-end sequencing was performed on the HiSeq4000 sequencer. Raw RNA-Seq data were aligned to the Human reference genome (Version hg19 from UCSC) using the STAR (V 2.4.2) aligner (28). Aligned reads were quantified against the reference annotation (hg19 from UCSC) to obtain Fragments per Kilobase per million (FPKM) and raw counts using Cufflinks (v.2.2.1) and HTSeq, respectively (29, 30). Differential expression was performed on raw counts with the limma package in R (31). Principal component analysis was performed on the log2-transformed FPKM expression values in R statistical software. Gene set enrichment analysis (GSEA) was performed using software from Broad Institute. Genes were ranked by the t-statistic value and used to identify significantly enriched biological pathways. Differential expression was performed, and expression profiles of synergistic (EOB > 20) versus nonsynergistic (EOB < 20) cell lines were compared.

**metaVIPER analysis**

The Virtual Inference of Protein-activity by Enriched Regulon analysis (VIPER) algorithm is a computational systems biology approach to infer protein activity from gene expression profiling (32, 33). In the absence of an available cancer-type–specific regulatory network, metaVIPER (34) can be effectively used to infer protein activity.

All regulatory networks used for metaVIPER analysis were reverse engineered by ARACNe (35). Twenty-four core The Cancer Genome Atlas (TCGA) RNA-Seq–derived interactomes are publicly available in the R Bioconductor package aracne.networks (36). After standard read alignment of RNA-Seq data by STAR to the GRCh38 reference genome build and summarization of expression quantities at the gene count level, gene expression was normalized by the Variance Stabilization Transformation, as implemented in the DESeq2 package on Bioconductor (37). A gene expression signature was computed between each synergistic cell line versus the reference group of nonsynergistic cell lines using the viperSignature function in the VIPER package, followed by application of the analytic Rank-based enrichment analysis using each of the available interactomes (38, 39). Normalized enrichment scores are integrated by Stouffer’s method. Pathway analysis on the differential protein activity signature was performed using GSEA with “Cancer Hallmark” and “Gene Ontology” gene sets provided in the Broad MSigDB collections (40).

A machine-learning classifier for predicting synergy with GSK126 and romidepsin using basal protein activity signatures was developed after first running VIPER on scaled gene expression signatures, resulting in protein activity profiles for each sample. The random forest method was applied iteratively with the addition of anywhere from 1 to 100 of the most differentially active proteins between synergistic and nonsynergistic cell lines. For each split in the decision tree, the minimum of the number of proteins made available for classification of 5 was used. The random Forest algorithm was run with 1,000 iterations of 3-fold cross-validation to estimate the ROC.

**Results**

**GSK126 and romidepsin synergize in EZH2 dysregulated lymphomas**

To understand the effects of EZH2 inhibition and HDAC inhibition in cell lines with or without EZH2 dysfunction and HAT mutations, a panel of 21 lymphoma cell lines was exposed to GSK126, an EZH2 inhibitor, and romidepsin, a pan-HDAC inhibitor, as single agents. Both B-cell lymphoma and TCL were exposed to GSK126 and romidepsin over 72 hours. Low drug inhibitions (Fig. 2A), lymphoma cell lines were simultaneously treated with GSK126 and romidepsin for 24 to 48 hours and evaluated by flow cytometry (Fig. 2C and D; Supplementary Fig. S4). A time point prior to the maximum EOB value was selected in order to capture the events prior to complete cellular demise (24 hours for Pfeiffer, 48 hours for OCI-Ly7, SU-DHL-10, and SU-DHL-6). Increased apoptosis was observed with the combination as compared with single-agent exposure. Apoptosis was also confirmed by decreased procaspase 3 and increased PARP cleavage following exposure to the combination as measured by immunoblot (Fig. 2E). In addition, as compared with single-agent treatment, the level of p21 was increased after exposure to GSK126 and romidepsin (Fig. 2E).

**Coexpression to GSK126 and romidepsin leads to enhanced acetylation and hypomethylation of H3K27 as well as dissociation of the PRC2 complex**

To understand the effects of dual epigenetic targeting on both acetylation and methylation of histone, 4 GC-DLBCL cell lines were exposed to control, GSK126, romidepsin, or the combination. Treatment with GSK126 and romidepsin led to increased acetylation and decreased trimethylation of H3K27 as compared with single agents as detected by histone extraction and immunoblot (Fig. 3A). These findings were validated by mass spectrometry (Fig. 3B–E).
Protein levels of EZH2 and other members of PRC2 complex (SUZ12, EED, and RbAp 46/48) were significantly decreased after dual treatment with GSK126 and romidepsin compared with single-agent exposure (Fig. 3F). Coimmunoprecipitation pull-down with EZH2 demonstrated dissociation of the PRC2 complex after simultaneous exposure to GSK126 and romidepsin. Specifically, exposure to romidepsin alone or in combination with GSK126 led to dissociation of EZH2 from EED, RbAp 46/48, and AEBP2 as compared with control, suggesting that romidepsin directly contributes to the breakdown of the PRC2 complex (Fig. 3G). In addition, HDAC2 and DNMT3L were also found to disassemble from the EZH2–PRC2 complex after combination therapy. Mass spectrometry confirmed disappearance of members of the PRC2 complex from EZH2 (Fig. 3H and I). With this in mind, we hypothesized that romidepsin may be responsible for the disruption of the PRC2 complex through direct acetylation of one or more subunits of the complex. To evaluate this hypothesis, SU-DHL-10 cells were treated with romidepsin, and immunoprecipitation using acetyl-lysine antibodies was performed. Based on mass spectrometry analysis, a 2-fold increase estimated by spectral counts of RbAp 46/48 (RBBP4) was observed after exposure to romidepsin as compared with control (FDR < 1.0%; Fig. 3J). Taken together, this suggests that the disruption of the PRC2 complex was secondary to direct acetylation of RbAp 46/48, which is responsible for PRC2 complex recruitment to nucleosomes (41).

Figure 1.
Sensitivity to GSK126 is predicted by the presence of EZH2 mutation. A, Single-agent GSK126 cell viability curves of 21 lymphoma cell lines (Blue, GC-DLBCL; Red, ABC-DLBCL; Green, T-cell lymphoma; and Black, Mantle cell lymphoma) after 144-hour exposure. B, Cell viability curves for 21 lymphoma cell lines exposed to romidepsin at 72 hours. C, IC50 values (144 hours) for respective cell lines after exposure to GSK126. Dysfunction is defined as overexpression (OE) of EZH2 and mutated EZH2 combined. D, IC50 values after 72-hour exposure to romidepsin. There is a trend toward the presence of HAT mutation and sensitivity to romidepsin (P = 0.05). Experiments were performed in triplicates and performed 3 times.
Figure 2.
GSK126 and romidepsin are synergistic in EZH2 dysregulated lymphomas. A, Given the frequency of EZH2 mutations along with HAT mutations, dual inhibition of these deregulated pathways may serve as a rational method to reverse transcriptional repressed state. B, 72-hour coexposure of GSK126 (G) and romidepsin (R) leads to potent synergy in lymphoma cell lines with EZH2 dysregulation as measured by EOB. C–F, G plus R induces apoptosis in GC-DLBCL cell lines at 48 hours as demonstrated by flow cytometry. G, G plus R leads to increased levels of p21, in turn, leading to apoptosis of GC-lymphomas as depicted by cleavage of PARP and increased pro–Caspase-3 levels. Experiments were performed in triplicates and performed 3 times.
Figure 3.
Combination of GSK126 and romidepsin leads to decreased methylation and increased acetylation of H3K27 and dissociation of the PRC2 complex. A, Acetylation of H3K27 was increased after combination therapy as compared with single-agent therapy. H3K27me3 decreased after the combination. B–E, Mass spectrometry confirms acetylation and methylation findings in 2 GC-DLBCL cell lines. Fold change calculated relative to control. F, Protein levels of EZH2 and PRC2 complex members are decreased after exposure to GSK126 and romidepsin as compared with single-agent therapy. G, Coimmunoprecipitation after treatment with GSK126, romidepsin, or the combination demonstrates dissociation of the PRC2 complex members after exposure to the combination of GSK126 and romidepsin. H and I, Mass spectrometry after 24-hour exposure to romidepsin (2.5 nmol/L) in SU-DHL-10 cells demonstrates dissociation of EZH2-SUZ12 from the PRC2 complex. J, Mass spectrometry after co-immunoprecipitation (co-IP) with acetyl lysine antibody demonstrated a 2-fold increase estimated by spectral counts between untreated SU-DHL-10 cells and romidepsin-exposed cells. Using Proteome Discoverer 2.1, this acetylated protein was identified as RbAp 46/48 (RBBP4; FDR < 1.0%).
HDAC2 plays a critical role in the synergy between GSK126 and romidepsin.

Based on the finding that HDAC2 dissociated from PRC2 complex after dual inhibition of EZH2 and HDACs (Fig. 3G), direct targeting of HDAC2 using a selective HDAC 1/2 inhibitor, ACY957 (42), was combined with GSK126 and was found to be synergistic (Fig. 4B). HDAC2 shRNA constructs were developed in order to confirm the role of HDAC2 inhibition in the synergy between GSK126 and romidepsin. Increased acetylation of H3K27 was found in HDAC shRNA HEK 293T cells, mimicking the effects of romidepsin, which was further enhanced by treatment with GSK126 (Fig. 4C). Decreased methylation of H3K27 was more pronounced in HDAC2 shRNA cells treated with GSK126, mirroring the effects of GSK126 and romidepsin exposure. Single-agent GSK126 exposure in HEK 293T cells did not significantly change the status of acetylation or methylation of H3K27.

GSK126 and romidepsin lead to improved OS and tumor growth delay in an in vivo mouse xenograft model.

A SU-DHL-10 mouse xenograft model was selected due to the fact that SU-DHL-10 represents a GC-DLBCL cell line that harbors an EZH2-activating mutation as well as HAT mutations (CREBBP and EP300). Mice were exposed to control, GSK126, romidepsin, or the combination as detailed in Fig. 5A. The combination was well tolerated in mice with no appreciable change in weight (Fig. 5B). Compared with single-agent exposure, dual therapy with GSK126 and romidepsin led to significant tumor growth delay (P < 0.05) and increase in OS (P < 0.0001; Fig. 5C and D). Moreover, pretreatment with GSK126 for 1 week did not improve...
Figure 5.
Combination of GSK126 and romidepsin improves OS in a mouse xenograft model. A, Treatment schema. Combination arm received GSK126 on days 1, 4, 8, 11, 15, and 18; romidepsin dosed on days 1, 8, and 15. B, Combination of GSK126 and romidepsin is tolerable as demonstrated by stability of weight. C, Coexposure to GSK126 and romidepsin leads to improved tumor control compared with single-agent GSK126 or romidepsin. D, Combination of GSK126 and romidepsin leads to improved OS compared with single-agent GSK126 or romidepsin. E, PK/PD parameters after single i.p. injection of GSK126 and romidepsin. Intratumor GSK126 continues to increase over time and is still present at 24 hours. F, Serum romidepsin pharmacokinetic parameters after single injection of GSK and romidepsin over 24 hours.

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Table 1.
Pharmacokinetic parameters of GSK126 and romidepsin in serum and tumor samples. Median Cmax of GSK126 was 1657.5 ± 413.6 ng/mL, which translates to 3.15 μmol/L (in vitro IC₅₀ of GSK126 in SU-DHL-10 is 0.7 μmol/L), whereas romidepsin was 98.24 ± 62.50 ng/mL or 0.18 μmol/L (in vitro IC₅₀ of romidepsin in SU-DHL-10 is 2.59 nmol/L; Fig. 5E and F). The serum AUC₀–last of GSK126 and romidepsin were 2828.57 (h·ng/mL) and 5.51 (h·ng/mL), respectively. The intratumor concentration of GSK126 increased over time, whereas the...
Many groups have demonstrated that gene expression profiles can be used to develop robust classifiers to predict drug sensitivity, but are difficult to validate in new datasets due to the inherent noise of RNA expression measurements and the risk of false discovery (33). In contrast, VIPER inference of protein activity is highly reproducible and biologically relevant. We developed a random forest classifier from the basal protein activity profiles of this diverse set of lymphoma cell lines to predict synergy between GSK126 and romidepsin. This classifier demonstrated good ROC on 3-fold cross-validation, with an AUC of 0.89 and an accuracy rate of 0.83 for predicting synergy (Supplementary Fig. S7). The classifier plateaued in performance with the inclusion of only 8 proteins (NDUFA13, CREB3R, MRPL12, KAT2B, ASF1B, BMPR2, POLR1I, and IL6ST), consistent with the ability of VIPER to identify biologically relevant proteins. Interestingly, decreased activity of KAT2B, an important HAT protein, was one of the most prominent features in the classifier for predicting synergistic activity of GSK126 and romidepsin.

Discussion

Epigenetic alterations have been implicated as drivers of lymphomagenesis, with EZH2 dysregulation and HAT inactivating mutations being central to the pathogenesis of GC-DLBCL. Given the prominence of EZH2 dysregulation in lymphoma, selective EZH2 inhibitors have been developed and have shown single-agent activity in early clinical studies (13, 43). Individually, mutations in EZH2 and HAT produce a repressed transcriptional state, and together, the PRC2 complex recruits HDAC 1/2, leading to additional transcriptional repression. In this context, dual inhibition of EZH2 and HDACs may serve as a rational therapeutic platform in lymphomas harboring epigenetic derangements (Fig. 2A). We describe that the combination of GSK126 and romidepsin was synergistic in EZH2 dysregulated lymphoma cell lines secondary to disassembly of the PRC2 complex due to acetylation of RbAP 46/48. This in turn caused attenuation of H3K27 methylation, increased acetylation, and upregulation of p21, which together triggered apoptosis. Acetylation of tumor suppressors and oncogenes has been well described (16, 44). EZH2 has been shown to be directly acetylated by P300/CRBP-associated factor (PCAF) and deacetylated by SIRT1 in lung adenocarcinoma models, with acetylation of EZH2 having no effects on EZH2’s ability to interact with other members of the PRC2 complex (45). Acetylation of EZH2 itself was not identified in our studies, however, we demonstrate that exposure to GSK126 and romidepsin leads to acetylation of RbAP 46/48, in turn, causing instability of the PRC2 complex, preventing EZH2 from catalyzing trimethylation, and leading to an open chromatin state.

Xenograft experiments demonstrated improvement in OS and tumor growth delay favoring the combination arm. Interestingly, intratumor concentrations of romidepsin were below the level of detection after cotreatment with GSK126 and romidepsin, which we have observed in prior combination studies (25). However, despite the undetectable intratumor concentration of romidepsin, intratumor concentrations of GSK126 increased over time, with the combination arm demonstrating potent synergy compared with single-agent therapy as manifested by increased OS and delayed tumor growth kinetics. Although complete tumor shrinkage was not observed in our xenograft studies, SU-DHL-10 has a very high proliferative rate owing to the fact that it harbors...
Figure 6.
Synergistic cell lines share a common basal gene and protein signature. **A,** Synergistic (EOB/C21 > 20) cell lines display a common basal gene expression signature, with upregulated genes such as HDAC9, AHCY, and MBD3. Genes are fully listed in Supplementary Fig. S5. **B,** Synergistic cell lines share enrichment in epigenetic pathways. **C** and **D,** Using Meta-VIPER, synergistic cell lines are enriched in pathways involving cell-cycle control, DNA replication, and chromatin remodeling with downregulation of differentiation and inflammatory pathways. **E,** Unbiased interrogation of 400 proteins revealed cosegregation of several proteins with EZH2 including HDAC1/2, DNMT3B, and MYC in primary patient samples (TCGA).
translocations of both MYC and BCL2 classifying it as a double hit lymphoma (46). Double hit lymphomas are most frequently of GC origin and are notoriously clinically challenging as patients often relapse after first-line therapy and salvage chemotherapy (47). Thus, our data may suggest a role of dual inhibition of EZH2 and HDACs for the treatment of double hit lymphomas. Given there has been limited success in identifying targeted therapy for double hit lymphomas, this warrants further investigation.

With the use of next-generation sequencing, individualized approaches to cancer therapy may arise based on unique gene expression patterns and mutational profiles that collectively contribute to a specific molecular phenotype. In an effort to identify a gene expression profile that may select patients that would benefit from dual EZH2 and HDAC inhibition, pretreatment RNA sequencing on a panel of lymphoma cell lines was performed. Cell lines demonstrating synergy to combined epigenetic therapy share a common basal genetic signature with enrichment in chromatin remodeling and gene silencing pathways, with identification of 69 genes that are expressed in a similar pattern. Using metaVIPER, enrichment of chromatin modification and epigenetic pathways was verified, but it also identified enrichment of DNA repair/synthesis and cell-cycle regulation pathways as well as downregulation of immune/inflammatory pathways in synergistic cell lines as compared with nonsynergistic cell lines. Interestingly, recent evidence suggests that EZH2 and DNM1T inhibit tumor cell production of T helper 1 type cytokines CXCL9 and CXCL10 as well as infiltration by effector T cells, all of which can be reversed by inhibition of EZH2 and DNM1T (48). Correlative studies to characterize the tumor T-cell infiltrate in the context of pre- and on-treatment biopsies after treatment with EZH2 inhibitor in conjunction with HDAC inhibitor would further assist in understanding these observations. Therefore, a phase I/II clinical trial investigating this novel combination with extensive biological correlatives is in development.

In line with the shift toward precision medicine, recent genomic analysis of primary DLBCL patient samples has led to two new proposed DLBCL classification systems, including an "EBZ signature" characterized by EZH2 mutations and BCL2 translocations (49), and a "cluster 3" subgroup identified by BCL2 mutations in conjunction with KMT2D, CREBBP, and EZH2 dysregulation (50). The identification of a DLBCL molecular subtype, predominately of GC origin, characterized by EZH2 mutations and BCL2 abnormalities in conjunction with the data presented here suggests that the addition of a BCL2 inhibitor to the combined inhibition of EZH2 and HDACs may be synergistic. This ultimately requires further investigation.

Our findings provide the biological rationale and lay the groundwork for a future clinical trial of targeted epigenetic therapy in GC-DLBCL. The combination of dual EZH2 and HDAC inhibition first-line therapy may potentially serve as a precision medicine therapeutic platform in lymphomas derived from the GC and those harboring an epigenetically repressed transcriptional state.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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