EZH2 inhibition by tazemetostat results in altered dependency on B-cell activation signaling in DLBCL

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Abbreviations list:
ANT – Antagonism
SYN – Synergy
N/E – No effect
NHL – Non-Hodgkin’s Lymphoma
WT – Wild Type
BCR – B-cell receptor
DLBCL – Diffuse Large B-Cell Lymphoma
GCB – Germinal Center B-cell
ABC – Activated B-cell
GRAG – Glucocorticoid Receptor Agonist
TAZ – Tazemetostat
GSEA – Gene Set Enrichment Analysis
FDR – False Discovery Rate
FL – Follicular Lymphoma
MZL – Marginal Zone Lymphoma
PMBL – Primary Mediastinal B-Cell Lymphoma
Abstract

The EZH2 small molecule inhibitor tazemetostat (EPZ-6438) is currently being evaluated in phase II clinical trials for the treatment of non-Hodgkin’s Lymphoma (NHL). We have previously shown that EZH2 inhibitors display an anti-proliferative effect in multiple pre-clinical models of NHL, and that models bearing gain-of-function mutations in EZH2 were consistently more sensitive to EZH2 inhibition than lymphomas with wild-type (WT) EZH2. Here, we demonstrate that cell lines bearing EZH2 mutations show a cytotoxic response, while cell lines with WT-EZH2 show a cytostatic response and only tumor growth inhibition without regression in a xenograft model. Previous work has demonstrated that co-treatment with tazemetostat and glucocorticoid receptor agonists lead to a synergistic anti-proliferative effect in both mutant and wild-type backgrounds, which may provide clues to the mechanism of action of EZH2 inhibition in WT-EZH2 models. Multiple agents that inhibit the B-cell receptor pathway (e.g. ibrutinib) were found to have synergistic benefit when combined with tazemetostat in both mutant and WT- EZH2 backgrounds of diffuse large B-Cell Lymphomas (DLBCL). The relationship between B-cell activation and EZH2 inhibition is consistent with the proposed role of EZH2 in B-cell maturation. To further support this, we observe that cell lines treated with tazemetostat show an increase in the B-cell maturation regulator, PRDM1/BLIMP1 and gene signatures corresponding to more advanced stages of maturation. These findings suggest that EZH2 inhibition in both mutant and wild-type backgrounds leads to increased B-cell maturation and a greater dependence on B-cell activation signaling.
Introduction

Disruption of chromatin modulation is emerging as an important, if not requisite, step in the process of oncogenesis. Mutations in chromatin modifiers are frequent occurrences in a number of cancers and are often associated with aberrant cell fate decisions (1-3). These lesions are particularly frequent events in non-Hodgkin's B-cell Lymphoma. Loss-of-function mutations in EP300, CREBBP or KMT2D (MLL2) or gain-of-function mutations in EZH2 occur frequently in B-cell lymphomas (4-6). Recent reports have demonstrated that loss of KMT2D or EZH2 gain-of-function leads to disruption of cell fate decisions (e.g. differentiation or apoptosis) and responses to extracellular signaling (7-10). Loss of cell fate control allows lymphomas to remain in a highly proliferative state and not proceed through the normal B-cell maturation process. For instance, germinal center lymphomas (follicular, GCB-DLBCL) maintain a transcriptional program that supports autonomous proliferation (11). For normal B-cells, the germinal center reaction is maintained by a gene expression program that is largely governed by the transcriptional repressor, BCL6 (12). During this phase, cells undergo a period of rapid proliferation, somatic hyper-mutation, reduced DNA damage response and class switch recombination. B-cells that successfully produce a high affinity B-cell receptor-antigen interaction will ultimately undergo activation and differentiation; those that fail to produce a B-cell receptor that recognizes antigen or that recognizes a self-antigen will undergo apoptosis via tightly regulated signaling pathways (11,12). In addition to B-cell receptor activity, many other pathways such as Toll-Like Receptor (13), cytokine (14) and CD40 signaling contribute to B-cell activation, differentiation and apoptosis regulation (11), and these responses are frequently altered in DLBCL (15). While follicular and GC lymphomas often have many characteristics of germinal center B-cells, they typically lose the ability to undergo apoptosis and maturation (15).

BCL6, a known regulator of B-cell maturation is mis-regulated by translocations with the 3q27 locus, resulting in its over-expression, an enhancement of the germinal center program, and a subsequent deficiency in B-cell differentiation. Additional genetic lesions, including mutations in BCL6, MEF2B, and FBX011 have been reported to enhance the ability of BCL6 to maintain the germinal center phenotype. These mutations lead to enhanced BCL6 stability or altered activity (12,16,17). Defective apoptosis and differentiation allow B-cells to remain in the germinal center reaction indefinitely which results in a germinal center lymphoma (16). In addition to GCB lymphomas, it has been shown that gain-of-function in Bcl6 frequently occurs in ABC lymphomas (6), which display a more mature, post germinal center phenotype. The role of BCL6 in ABC lymphomas remains largely unknown and it is possible that its alteration is required only for initiation of this lymphoma subtype (18), although recent reports indicate that activated B-cell lymphomas may require sustained BCL6 activity (19). As above, KMT2D and CREBBP are frequently mutated in FL and DLBCL and have also been implicated in B-cell maturation specifically via a reduction in CD40L and cytokine responsive transcription (8,9,20). The loss of chromatin modifiers in multiple lymphoma subtypes indicates that chromatin regulation may be critical at multiple points along the B-cell lineage. In contrast, EZH2 gain-of-function mutation is found almost exclusively in follicular lymphoma and GCB DLBCL (6), suggesting that increased EZH2 activity does not allow cells to progress past the germinal center. In addition, ABC lymphomas also have unique lesions, such as loss of PRDM1, which prevent lymphomas from progressing beyond the plasmacytic phenotype (21).
Consistent with the importance of chromatin modifications in B-cell maturation, recent reports have demonstrated that EZH2 activity is required for cells to maintain the germinal center reaction. These experiments have shown that loss of EZH2 prevents the formation of germinal centers in mouse lymph nodes. Moreover, knock-in of gain-of-function mutations of EZH2 occurring primarily at the Y646 residue have been shown to induce or contribute to lymphomagenesis in mouse models (7,10). It was recently discovered that treatment of normal B-cells or DLBCL cell lines with small molecule inhibitors of EZH2 can induce several hallmarks of B-cell maturation (immunoglobulin and PRDM1 induction) (7,10,22) and that B-cell maturation in the lymph node is controlled by the transcriptional repressive effects of BCL6 and EZH2 (23). These reports are consistent with many studies that have explored the effects of EZH2 inhibitors in cell lines derived from B-cell lymphomas. Many B-cell lymphoma cell lines are unaffected or show only a modest decrease in proliferation or cell viability in response to EZH2 inhibition, while cell lines with mutant EZH2 generally show a more robust response than their wild-type counterparts (24,25).

In this report, we explore a variety of drug combinations with tazemetostat (EPZ-6438) to determine if its anti-lymphoma activity can be enhanced in cancers with wild-type EZH2. Tazemetostat was previously shown to have a synergistic relationship with glucocorticoid receptor agonists (GRAGs) in co-treatment models of DLBCL (26). This work showed that cell lines with wild-type EZH2 were sensitive to the tazemetostat/GRAG combination, while only showing modest effects with single agent tazemetostat. To expand on this effort, here we explore combination effects with clinically and biologically relevant agents that target B-cell biology. We find that agents which inhibit B-cell activation (BTKi, SYKi, GRAGs) show a strong synergistic relationship with tazemetostat. In contrast, we find that tazemetostat is less cytotoxic when combined with agents that activate B-cells (LPS, CD40L). Consistent with previous studies demonstrating a role for EZH2 in B-cell differentiation, we observe that co-treatment of cell lines with CD40L and tazemetostat leads to an upregulation of the B-cell maturation regulator PRDM1, while no effect was observed for either agent individually. Taken together, these results suggest that EZH2 inhibition leads to a cell fate decision governed by B-cell activation.

**Materials and Methods**

**Compounds**

Dexamethasone (S1322), Everolimus (S1120), Ibrutinib (S2680), Idelalisib (S2226), MK-2206 (S1078) (27), Prednisolone (S1737), R406 (Tamatinib, S2194) (28), and Trametinib (S2673), were all obtained from Selleck Chemicals (Houston, TX). APRIL (5860-AP), CD40 ligand (6420-CL), and BAFF/TNFSF13B (2149-BF) were all obtained from R&D Systems (Minneapolis, MN). LPS (L4391) was obtained from Sigma-Aldrich (St. Louis, MO). F(ab’)2 Anti-human IgM+IgG (16-5099-85) was obtained from Ebioscience (San Diego, CA). Tazemetostat was synthesized as described previously. (29)

**Cell Culture**

All cell lines used in this manuscript were obtained from the following sources between 2011 and 2016. No cell line was passaged for more than 6-months prior to an experiment. Culture of diffuse large B-cell lymphoma cells WSU-DLCL2, SU-DHL-10, Toledo, and OCI-LY19 was previously described (24,30).
KARPAS-422 (ACC 32), OCI-LY7 (ACC 688), SU-DHL-5 (ACC 571), and OCI-LY3 (ACC 761) were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Germany). SU-DHL-6 (CRL-2959), HT (CRL-2260), Farage (CRL-2630), and SU-DHL-2 (CRL-2956) were obtained from ATCC (American Type Culture Collection, Manassas, MD USA). TMD8 was obtained from Tokyo Medical and Dental University (Tokyo, Japan). No authentication information for TMD8 is available. HT, Farage, SU-DHL-5, TMD8, KARPAS-422, SU-DHL-6, OCI-LY3 and SU-DHL-2 were maintained in RPMI supplemented with 10% fetal bovine serum and cultured in a humidified atmosphere including 5% CO2. OCI-LY7 is maintained in IMDM supplemented with 20% fetal bovine serum and cultured in a humidified atmosphere including 5% CO2. Mutations in EZH2, MYD88 and CD79B in cell lines were evaluated using the CCLE (https://portals.broadinstitute.org/ccle/home) and Cosmic (http://cancer.sanger.ac.uk/cosmic) databases.

**RNA Seq**

RNA seq was executed by Expression Analysis (http://www.q2labsolutions.com/genomicslaboratories). RNA was converted into cDNA libraries using the Illumina TruSeq Stranded mRNA sample preparation kit (Illumina # RS-122-2103). RNAseq was performed on the Illumina HiSeq platform using 50 base pair, paired end sequencing. Approximately 30 million reads were collected. Data quality was evaluated using spike-in controls. ERCC controls from Ambion® via Life Technologies™ (Grand Island, NY USA). 92 oligos were added to cover a 106-fold concentration range. Genome alignments of reads were performed using STAR software version 2.4 and were quantified using RSEM version 1.2.14. Upper quartile normalized values were used for subsequent analysis. RNAseq data sets were analyzed using GSEA software (Broad Institute). Normalized RNAseq values from samples with and without tazemetostat treatment were run through the algorithm. Altered genes were compared against gene sets found to be induced in the transition from germinal center B-cells to memory B-cells (31).

**CHIP Seq**

Cells were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield.

An aliquot of chromatin (25 or 30 ug) was precleared with protein A agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using antibodies against H3K4me3 (Active Motif – 39159), K3K27me3 (Cell Signaling Technology – 9733), and EZH2 (Active Motif – 39901). Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation.
Quantitative PCR (QPCR) reactions were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). The resulting signals were normalized for primer efficiency by carrying out QPCR for each primer pair using Input DNA.

Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on Illumina’s NextSeq 500 (75 nt reads, single end). Reads were aligned to the human genome (hg19) using the BWA algorithm (default settings). Duplicate reads were removed and only uniquely mapped reads (mapping quality >= 25) were used for further analysis. Alignments were extended in silico at their 3'-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic “signal maps”) were stored in bigWig files. Data was visualized using UCSC genome browser (https://genome.ucsc.edu).

**In Vitro Cellular Assays**

For long term proliferation assays, seeding densities for each cell line are provided in Table S1. On day 0, cells were seeded in T75 flasks and either treated with 1 µM tazemetostat, 0.25 µM tazemetostat, or DMSO. Cells were split and reseeded every 3-4 days. 11-day IC50 determination was performed as described previously (32). To determine the combinatorial effects of tazemetostat with other compounds, lymphoma cells were treated according to a 4+3 treatment model (4-day pretreatment with tazemetostat followed by a 3-day co-treatment with tazemetostat and compound of interest) or in the case of Toledo cells only, to a 6+5 treatment model (6-day pretreatment with tazemetostat followed by 5-day co-treatment with tazemetostat and compound of interest). For combination studies of tazemetostat and biologics in the SU-DHL-10 cell line, a 4-day co-treatment model was established due to rapid apoptosis following single agent tazemetostat treatment.

Lymphoma cells were seeded into flasks (based on the densities shown in Table S1) and pre-treated with 7 concentrations of tazemetostat or DMSO for 4 days (with the exception of Toledo cells, that were pretreated for 6 days and Farage cells, that were pretreated for 7 or 8 days). Cells were then split and co-treated with tazemetostat and a second agent of interest using the HP D300 digital dispenser (Tecan Group, Mannedorf, Switzerland) in 96-well plates for additional 3 days, with the exception of Toledo cells, that were co-treated for 5 days. SU-DHL-10 cells were plated treated directly in solid white 96-well plates and cotreated with tazemetostat and compounds of interest for 4 days. Doses of drugs used in these assays were near the IC50 for each agent (Table S2). Both agents were serially diluted two-fold and combined in a matrix with constant ratios diagonally across the plate with a final DMSO content of 0.11% (v/v). Three concentrations with a four-fold dilution were chosen to combine with tazemetostat. After 3 days cotreatment (5 days for Toledo assays), cell viability was measured via ATP content using CellTiter-Glo® (Promega, Madison, WI) and luminescence was detected using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

**In Vivo Models**
All of the procedures related to animal handling, care and the treatment in this study were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Chempartner following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Female CB17 SCID mice (6-8 weeks old) were obtained from Shanghai Lingchang Biotech Co (Shanghai, China). 1x10^6 OCI-LY19 and 5x10^6 Toledo cells were mixed with matrigel and inoculated subcutaneously in the right flank. Due to high animal to animal variability, SU-DHL-5 cells were sub-cultured from a previously established tumor. 5x10^6 of the explanted SU-DHL-5 cells were also inoculated in 50% matrigel into the right flank. Tazemetostat was suspended in 0.5% NaCMC with 0.1% Tween-80. Eight tumor bearing animals were used per dose group. Animals were treated with 125 mg/kg or 500 mg/kg twice daily. Tumor volume was measured every three or four days for the duration of the study. Mice were taken off study and euthanized once tumor measurements exceeded a volume of 2000 mm^3. Mean tumor volumes were calculated with SEM values (N=8).

**Apoptosis Analysis**

Apoptosis analysis was performed as shown previously (32). All cells were seeded at densities that ensured logarithmic growth for each segment of treatment. Seeding densities are shown in Table S1. For combinations with ibrutinib, KARPAS-422, Farage, SU-DHL-5, and SU-DHL-2 cells were treated with DMSO or 1 µM of tazemetostat for 4 days. Cells were split and retreated with DMSO or 1 µM tazemetostat, 3 µM ibrutinib, or in combination with tazemetostat and ibrutinib at those concentrations for 3 additional days. Cells were harvested on days 4 and 7 to allow for analysis of Annexin V staining. For the CD40 ligand combinations, KARPAS-422 cells were treated with DMSO or 1 µM of tazemetostat for 4 days. Cells were split and retreated with DMSO, 1 µM tazemetostat, 100 ng/mL CD40 ligand or with the combination of both agents for 3 additional days. Cells were split one additional time in the same manner above for 3 more days, for a total of 10 days in treatment. Cells were harvested on days 4, 7, and 10 to allow for analysis of Annexin V staining. In apoptosis experiments, statistical analysis was performed using one-way ANOVA plus Bonferroni’s post-test where ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, ns P>0.05.

**Combination synergy quantitation**

Concentration response plots were generated in GraphPad Prism version 7.0 for Windows, GraphPad Software, (La Jolla, California) and curves fitted to a four-parameter logistic model with variable slope (2 biological replicates). Fraction affected (Fa), a measure of compound inhibition was calculated at each treatment concentration by the following equation: Fa = 1-((RLU cmpd-RLU min)/(RLU max- RLU min )), where RLU = Relative Luminescence Units, RLU cmpd = value for test treatment, RLU max is the maximum signal control (DMSO treatment) and RLU min is the minimum signal control (positive control or full inhibition control). The 50% inhibitory concentrations were interpolated from the 4-parameter logistic model as the concentration that achieved Fa = 0.5. Analysis of combinatorial effects and synergy quantification were performed by different methods according to the single agent activities of the combination partners: when both compounds had a calculable IC50 value then the Combination Index (CI) was derived by the Chou-Talalay method (33) using the CalcuSyn for Windows version 2.1 software.
Combinations with CI < 1 demonstrated synergy (SYN), those with CI > 1, antagonism (ANT), and those with CI = 1 additivity (ADD). When one of the combined agents did not reach an IC50, the extent of the combination benefit was determined by maximal fold shift in the IC50 of the other agent as a function of the concentration of the second agent compared to DMSO alone. Combination benefit or enhancement was demonstrated when the presence of the second agent produced a decrease of the IC50 value greater than two fold. In contrast, antagonism was demonstrated by a rightward shift of the IC50 greater than two fold. If no fold shift was observed, then it was denoted as no effect (N/E). If neither combined agent had a calculable IC50, CHALICE software (Horizon Discovery, Cambridge, UK) was used to calculate the Loewe volume (VLoewe) (34). A positive Loewe volume denoted synergy, and a negative Loewe volume, antagonism. A volume between -1 and 1 was denoted as additivity.

**Western Blotting**

Whole-cell lysates were prepared using RIPA buffer [10X RIPA Lysis Buffer, Millipore; 20-188], supplemented with 0.1% SDS, and protease inhibitor tablet [Sigma Aldrich; 4693124001]. Cells were pelleted, washed with ice-cold PBS, resuspended in ice-cold RIPA buffer, and incubated on ice for 5 minutes. Lysates were then sonicated 3 times for 5 seconds and incubated on ice for 10 minutes. Lysates were centrifuged at maximum speed for 10 minutes at 4°C. Protein concentrations were determined by BCA (Pierce; 23225). Twenty micrograms were loaded onto gels for PRDM1/Blimp-1/PRD1-BF1 (CST; 9115) analysis, 5 micrograms for H3K27me3 (CST; 9733), and 25 micrograms for IκBα (CST; 4814). β-actin (CST; 3700) and GAPDH (Millipore; MAB374) were used as protein loading controls. Lysates were fractionated on 4-12% Bis-Tris gel (Life technologies; WG1402BX10), transferred using iBlot (7 minutes on program 3, using nitrocellulose transfer stacks), and probed antibodies in Odyssey blocking buffer (Odyssey; 927-40000).

**Quantitative PCR**

All cells were seeded at densities that ensured logarithmic growth for each segment of treatment. Seeding densities can be seen in supplementary Table S1. All cells were treated with DMSO or 1 µM tazemetostat for 4 days. Cells were split and retreated with DMSO or 1 µM tazemetostat for 3 additional days. Cells were harvested and total mRNA was extracted from cell pellets using the RNeasy Plus Mini Kit (Qiagen; 74134). cDNA was made using the RT2 First Strand Kit (Qiagen; 330401). RT-PCR was performed using ViiA 7 Real-Time PCR Systems (Applied Biosystems [AB]) TaqMan probe based qPCR was carried out using TaqMan Fast Advanced Master Mix (AB; 4444964) and TaqMan primer/probe sets for PRDM1 (Invitrogen; Hs00153357_m1). Gene expression was normalized to housekeeping gene, 18S (Thermo, 4319413E), and fold change compared to DMSO was calculated using the ΔΔCt method.

**Results**

**Effects of Tazemetostat in Wild-Type vs. Mutant EZH2 Cell Lines**
Treatment of select B-cell lymphoma cell lines in culture with EZH2 inhibitors has been shown to decrease their proliferation and in some cases induce apoptosis. We and others have demonstrated that cell lines bearing gain-of-function mutations in EZH2 are significantly more sensitive to EZH2 inhibition (24,25). As we have previously published, lymphoma cell lines with mutant EZH2 are several orders of magnitude more sensitive to EZH2 inhibitors, as measured by IC$_{50}$ in an 11-day proliferation assay (Figure 1B). However, we observe decreases in proliferative activity in the majority of B-cell cancer cell lines tested, regardless of EZH2 mutation status, cell of origin or lymphoma subtype. As shown in Figure 1B, EZH2 wild-type cell lines with canonical ABC mutations like OCI-LY3 (MYD88), TMD8 (CD79B) display similar sensitivity to tazemetostat with EZH2 wild-type cell lines of GCB origin like Farage, OCI-LY7 or SU-DHL-5 (35). When comparing mutant and wild-type cell lines at identical concentrations of inhibitor, we observe that the EZH2 mutant cell line, KARPAS-422 shows a strong cytotoxic response at 250 nM tazemetostat while cell lines with wild-type EZH2, like SU-DHL-5 (GCB), Farage (GCB) and TMD8 (ABC) show dose dependent decreases in proliferation (Figure 1C). Moreover, the KARPAS-422 cell line shows an increase in apoptosis in response to tazemetostat treatment, while the three wild-type cell lines show little or none (Figure 1D). These findings suggest that mutant EZH2 cell lines have a strong dependency on EZH2 activity for their viability, while very little if any EZH2 activity is required for survival of wild-type EZH2 cell lines. These findings are consistent with previous work demonstrating that cytotoxicity occurs at very low doses of EZH2 inhibitor in mutant cell lines, but is not observed with any relevant concentration in wild-type cells (24,25).

While previous xenograft studies of mutant EZH2 lymphoma models have demonstrated sensitivity to tazemetostat (30), few studies have been carried out to evaluate response from models of wild-type EZH2. Three wild-type cell line xenograft models were developed to study this in greater depth. Animals with Toledo, SU-DHL-5 and OCI-LY19 cell line xenografts were treated with vehicle, or 125 or 500 mg/kg BID tazemetostat for their respective tumor growth windows. As shown in Figure 1E, significant dose-dependent tumor growth inhibition was observed in the OCI-LY19 model. However, the Toledo and SU-DHL-5 models showed only modest tumor growth inhibition that could not be readily differentiated from animal-to-animal variability from the vehicle control. Inhibition of H3K27me3 levels in SU-DHL-5 tumors and animal body weight changes are shown in Figure S1, demonstrating that dosing is sufficient and tolerated for this mouse strain. In contrast, previous work has shown that tazemetostat induces robust tumor regressions in multiple tumor models bearing EZH2 gain-of-function mutations (30).

**Tazemetostat creates a dependency on B-cell activation signaling**

The phenotypic differences in response to tazemetostat between wild-type and mutant EZH2 suggest that wild-type cell lines are able to compensate for loss of EZH2 activity and maintain viability. We further explored this observation by evaluating various agents for combination benefit with tazemetostat. Consistent with our previously published findings (26), we observed synergistic interactions between glucocorticoids and tazemetostat treatment in the majority of cell lines tested. While glucocorticoids have a number of effects on lymphoma cells, one of these effects is a reduction in NF-κB signaling (36) which is critical for B-cell activation. Based on this finding, we treated lymphoma cells with kinase inhibitors that target the B-cell activation pathway at various points along the B-cell
receptor signaling cascade. Figure 2 shows the effects of combinations of tazemetostat with ibrutinib (BTK inhibitor) or tamatinib (SYK inhibitor). Both of these inhibitors target signal transduction through the B-cell receptor pathway. We observed synergy between these agents and tazemetostat suggesting a relationship between EZH2 and B-cell activation. Moreover, agents targeting additional nodes of the B-cell receptor pathway, including PI3K, AKT, mTOR and MEK1 (Table 1) were also found to be synergistic with tazemetostat. The synergistic relationship between the B-cell receptor pathway and EZH2 inhibition suggests that loss of EZH2 activity makes cells more dependent on B-cell activation signals for survival. To test this more directly, we sought to determine if by artificially stimulating B-cell activation, the effects of tazemetostat would be antagonized. We found that addition of LPS, BAFF, APRIL or CD40L (all activators of B-cells) was able to antagonize the effects of EZH2 inhibition in multiple cell lines (Figure 2A, Table 1). It is important to note that extended treatment times were used for Toledo and Farage due to their growth rates and drug combinations in the SU-DHL-10 cell line were added simultaneously for only 4 days because of a rapid onset of apoptosis in response to single agent tazemetostat, providing a caveat to direct comparisons between all cell lines. CD40L showed the most consistent effect across cell lines, and importantly treatment with CD40L alone had no effect on proliferation of the majority of the cell lines (Figure S2). These results suggest that much of the cytotoxic and growth inhibitory effects of EZH2 inhibitor are due to insufficient compensatory B-cell activation signaling. To further explore this observation, RNAseq was performed on Farage, SU-DHL-5 and KARPAS-422 cell lines treated with 1 µM tazemetostat for 4 days. Gene set enrichment analysis (GSEA) comparison of cell lines with and without tazemetostat treatment shows a decrease in expression of a BCR responsive gene set (37) following EZH2 inhibition (Figure 2B) suggesting that EZH2 alters how cells respond to this signaling pathway.

Inhibition of both EZH2 and the B-cell Receptor Pathway Induces Apoptosis in Wild-Type Cell Lines

The strong synergy observed between B-cell Receptor pathway inhibitors and tazemetostat in cell viability assays suggested that combination of these agents may be inducing a cytotoxic effect rather than the cytostatic effect observed with single agent. To test this hypothesis, KARPAS-422, SU-DHL-5 and Farage cells, co-treated with tazemetostat and ibrutinib, were stained with Annexin V and 7-AAD to evaluate apoptosis. Combination of tazemetostat and ibrutinib in all three cell lines showed a significant increase in apoptosis than what was observed for either single agent treatment (Figure 3A-C). The most significant increase in apoptosis was observed in SU-DHL-5 cells which are not sensitive to either agent individually. To determine if B-cell activation can suppress the pro-apoptotic effect of tazemetostat in a mutant cell line, we measured apoptosis in KARPAS-422 cells co-treated with tazemetostat and CD40L. Addition of CD40L greatly reduced tazemetostat-induced apoptosis (Figure 3D), while having no effect on the ability of tazemetostat to reduce the levels of H3K27me3 (Figure 3E). We also examined whether upstream signaling through CD40 is affected by tazemetostat treatment; in Figure S3, we show that CD40L-induced IkBα degradation is not affected by tazemetostat treatment suggesting that EZH2 does not impact the CD40 signal transduction machinery.

Tazemetostat Induces Molecular Events of B-cell Differentiation

To better understand the effects of tazemetostat in wild-type EZH2 lymphomas, RNAseq studies were performed on mutant EZH2 GCB, wild-type EZH2 GCB and wild-type EZH2 ABC cell lines with and
without EZH2 inhibitor treatment. Consistent with previous reports (7,10), gene set enrichment analysis showed that genes associated with B-cell maturation are induced upon EZH2 inhibition. We find that enrichment in memory cell gene sets (31) can be observed regardless of EZH2 mutation status, cell of origin or in vitro IC₅₀. As shown in Figure 4A, KARPAS-422 (GCB EZH2 Mut), Farage (GCB EZH2 WT), SU-DHL-5 (GCB EZH2 WT), and TMD8 (ABC EZH2 WT) show increases in expression of this gene set. In addition, we also observed increases in the B-cell maturation regulator and tumor suppressor, PRDM1/BLIMP1. PRDM1 message (Figure 4B) and protein (Figure 4C) were increased most strongly in the EZH2 mutant cell line KARPAS-422, but increases were also observed in SU-DHL-5 and Farage. No increase in PRDM1 was observed in the TMD8 cell line, as PRDM1 is already expressed (Figures 4B and 4C), which is common in ABC lymphomas. However, our results suggest that the role of EZH2 in B-cell maturation is not limited to PRDM1 regulation, as the upregulation of the maturation gene expression signature is observed in TMD8s despite no observable change in PRDM1 (Figure 4A-C). Consistent with the up-regulation of PRDM1, a gene set corresponding to ABC-DLBCL (38) was induced in the three GCB cell lines tested Figure S4, indicating molecular events of maturation were occurring. Not surprisingly, this gene set was not increased in the ABC-DLBCL cell line TMD8 as these genes were likely already elevated, as TMD8 is an ABC-derived cell line.

Similar to a previous report (7), we observe that like many other genes involved in B-cell maturation, the PRDM1 locus is bivalent. In Figure 4D, we show CHIP-Seq data from the PRDM1 locus in the Farage cell line which shows that EZH2, H3K27me3 and H3K4me3 are all present at the PRDM1 promoter. To further evaluate the repression of PRDM1 by wild-type EZH2, we explored the effect of tazemetostat on known regulators of PRDM1 expression in the SU-DHL-5 cell line. One such inducer, CD40L is an important mechanism for T follicular helper cells to stimulate B-cell maturation and PRDM1 expression via NFκB activation. We therefore assessed recombinant CD40L-mediated PRDM1 induction in the SU-DHL-5 cell line in the presence or absence of tazemetostat. As seen in Figures 4E, tazemetostat has only a modest effect on PRDM1 levels in the SU-DHL-5 line as a single agent and neither CD40L and IL-21 affect PRDM1 protein levels in SU-DHL-5 cells (Figure S5), while they have been shown to induce PRDM1 expression in normal B-cells (39). However, we observe that treatment with combinations of CD40L and tazemetostat strongly up-regulate PRDM1 in SU-DHL-5s (Figure 4E). While its importance in normal B-cell differentiation has been established, addition of IL-21 to tazemetostat treatment in the SU-DHL-5 cell line had no additional effect on PRDM1 protein levels (Figure S5). These findings suggest that EZH2 suppresses PRDM1 levels in response to B-cell activation, which may be important for the anti-lymphoma effects of tazemetostat. In addition, gene set enrichment analysis showed an increase in CD40L-response genes with tazemetostat treatment alone in KARPAS-422, Farage, SU-DHL-5 and TMD8 cell lines (Figure S6). This is consistent with previous reports demonstrating that EZH2 suppresses CD40L and other B-cell maturation related pathways at the level of transcription (7).

Discussion

As described in the work presented here and in other studies (24,25), B-cell lymphoma cell line models bearing EZH2 mutations show a strong cytotoxic response to EZH2 inhibitors. In contrast, wild-type cell lines show much less dependence on EZH2 activity for survival and only a cytostatic response to
treatment. These findings suggest that wild-type *EZH2* lymphomas have additional active pathways that support their growth and survival. A previous study has demonstrated a synergistic relationship between tazemetostat and glucocorticoids in both wild-type and mutant *EZH2* DLBCL cell lines (26). We present here that in addition to glucocorticoids, agents that selectively target the B-cell receptor pathway, ibrutinib (BTKi) and tamatinib (SYKi) also have a synergistic relationship with tazemetostat. Moreover, several other inhibitors that target kinases involved in B-cell receptor signaling (AKT, PI3K, mTOR and MEK1) also show this effect. These findings suggest that *EZH2* inhibition creates a dependency on B-cell activation signaling for cell viability. This concept is further illustrated by the finding that the effects of single agent tazemetostat can be diminished by artificially activating B-cell lymphoma lines with CD40L, BAFF, LPS, or BCR ligation.

Based on previous studies describing the role of *EZH2* in B-cell differentiation (7,22,25), we speculated that the altered dependence on B-cell activation signaling observed in lymphoma cell lines is due to progression through the B-cell maturation pathway. Previous studies have demonstrated that *EZH2* is essential for the maintenance of germinal center B-cells and that the key oncogenic effect of *EZH2* gain-of-function mutations is to delay or block progression through this step of B-cell maturation. We demonstrate that while no significant cytotoxicity is observed in cell lines with wild-type *EZH2*, gene sets associated with memory B-cells and the B-cell differentiation transcription factor PRDM1/BLIMP1 are up-regulated following tazemetostat treatment. In addition, we demonstrate that in the *EZH2* wild-type cell line Farage, the *PRDM1* promoter is enriched for H3K27me3 and H3K4me3 suggesting that it’s bivalent and poised for expression. We also note that in the SU-DHL-5 cell line, combination of CD40L and tazemetostat induces *PRDM1* expression while neither treatment affects PRDM1 levels as a single agent. These findings suggest that *EZH2* suppresses *PRDM1* expression and that with the appropriate activation stimulus and decrease in *EZH2* activity; its expression can be induced. As B-cells mature, their requirements for activation signals change (15). This concept is consistent with the observation that addition of CD40L or other B-cell activating agents can support viability in cell lines that have an otherwise cytotoxic response to tazemetostat. The synergistic cytotoxic effect of tazemetostat with inhibitors of B-cell activation signaling and the synergistic induction of PRDM1 with CD40L suggest that *EZH2* inhibition “opens the door” for a cell fate decision for B-cell lymphomas regardless of *EZH2* mutation status. This model is illustrated in Figure S7.

The molecular events of differentiation observed in vitro following tazemetostat treatment in wild-type *EZH2* lymphoma cell lines and the effects of *EZH2* inhibition on normal B-cells in mice suggest that tazemetostat may have beneficial effects in lymphomas with either wild-type or mutant *EZH2*. Our findings show examples of molecular events of differentiation in cell lines with GCB characteristics but many of those changes also occur in an ABC cell line following tazemetostat treatment. This suggests that the effects of *EZH2* on B-cell maturation may not be limited to a single cell of origin. But more importantly, the effects of *EZH2* inhibitors on cell fate and the synergistic effects observed with inhibitors of B-cell activation in vitro provide multiple avenues for drug combinations in the future. To that end, tazemetostat is currently being evaluated as monotherapy in clinical studies that have included patients with either wild-type or activating *EZH2* mutations. Patients enrolled in these studies...
have also included those with FL, transformed FL, GCB DLBCL, ABC DLBCL, MZL and PMBL. In addition, there are also ongoing combination studies with R-CHOP and with prednisolone.

References


Table 1. In vitro combinatorial effects of tazemetostat with inhibitors of the B-cell receptor pathway and modulators of B-cell activation. In vitro assays were performed in the indicated models with 4 days pretreatment with tazemetostat, followed by 3 days of the indicated combination unless otherwise indicated. Synergy (SYN), antagonism (ANT), additivity (ADD) and no combination effect (N/E) were assigned by different methods according to the single agent activity of tazemetostat and combination partners as described in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Modality</th>
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<th>WT EZH2 GCB</th>
<th>WT EZH2 ABC</th>
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<tr>
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<td><strong>GRAG</strong></td>
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* wsu-dlcl2, karpas-422, ^su-dhl-10, su-dhl-6, oci-ly19, ^farage, oci-ly7, su-dhl-5, toledo, tmd8, oci-ly3, su-dhl-2
Figure 1: Lymphoma cell lines with EZH2 mutation have a cytotoxic response to EZH2 inhibition with tazemetostat, while wild-type cell lines show a cytostatic response.

A) Chemical structure of tazemetostat (EPZ-6438) B) EZH2 gain-of-function mutant cell lines are more sensitive to tazemetostat (TAZ) than EZH2 wild-type cell lines in vitro. 11-day proliferation IC50 values for 19 wild-type DLBCL cell lines (left) and 9 mutant DLBCL cell lines (right) shown on a log plot. Proliferation was assessed by viable cell counts. C) Mutant EZH2 KARPAS-422 cell line, wild-type EZH2 GCB cell lines Farage and SU-DHL-5 and the ABC cell line, TMD8 cell line were treated with TAZ over a 42-day time course showing varying degrees of anti-proliferative responses. Cell number was measured at the indicated time points. The viable cell count (y axis) in each panel is presented on a logarithmic scale. D) Early stage apoptotic cell death is observed in mutant EZH2 KARPAS-422 cells but not in wild-type cell lines treated for 10 days with 1 µM TAZ or DMSO as measured by Annexin-V. Green stacks represent percentages of cells in early stage apoptosis (means +/- S.D., n=2). **P<0.01 (ANOVA plus Bonferroni’s post-test) KARPAS-422 cells treated with TAZ compared with DMSO, * P<0.1 (ANOVA plus Bonferroni’s post-test) Farage cells treated with TAZ compared with DMSO. E) OCI-LY19, SU-DHL-5 and Toledo cell lines xenograft-bearing mice were treated with vehicle, 125 or 500 mg/kg BID tazemetostat by oral gavage for the indicated times. Eight tumor bearing SCID mice per group were treated with the indicated doses of tazemetostat once tumors reached a minimum volume of 100 mm3. Mean tumor volumes ± SEM (n=8) are plotted. Endpoint was reached when tumor volume exceeded 2000 mm3. *P<0.01, **P<0.05

Figure 2: DLBCL cell lines become dependent on activation signals upon treatment with tazemetostat.

A) SU-DHL-5 (EZH2 WT, GCB), KARPAS-422 (EZH2 mutant, GCB) and Farage (EZH2 WT, GCB) cell lines were pretreated with TAZ for 4 days followed by co-treatment with the indicated doses of modulators of B-cell signaling for additional 3 days. All dose response curves were plotted as fraction affected versus the logarithmic concentration of tazemetostat. Synergy was quantified for each combination by the Loewe method using the Chalice software. The calculated Loewe volume (VLoewe) for each combination and the 3-day single agent IC50 value of the combined drug are indicated on the respective graphs. All analysis methodologies are described in Materials and Methods. Synergistic anti-proliferative activity was observed when TAZ was combined with ibrutinib or tamatinib in all cell lines. CD40L in combination with TAZ showed antagonism in KARPAS-422 and SU-DHL-5 but had no effect in Farage. B) BCR-ligation responsive gene sets decrease in response to tazemetostat treatment. GSEA was performed on RNAseq data from the indicated cell lines with and without tazemetostat treatment for a gene set corresponding to genes up-regulated following anti-BCR ligation in normal B-cells (37). Anti-BCR-induced genes were decreased in all three cell lines as indicated by NES scores. FDR < 0.001 for each comparison.

Figure 3: Inhibitors of B-cell activation cooperate with tazemetostat to induce apoptotic cell death in GCB cells. A-C) Cells were treated for 7 days with single agents (1 µM TAZ or 3 µM ibrutinib) or in combination. A significant increase of apoptosis was observed with the combination of TAZ and ibrutinib in GCB cells lines compared to the single agents (**P<0.01, ***P<0.001, ****P<0.0001 (ANOVA plus Bonferroni’s post-test). D) KARPAS-422 cells were treated for 10 days with single agents (1 µM TAZ or 100 ng/mL CD40L) or in combination. A significant increase of apoptosis is seen with single agent TAZ
compared to DMSO and a significant decrease of apoptosis is seen with the combination of TAZ and CD40L compared to TAZ single agent treatment (*P<0.1 (ANOVA plus Bonferroni’s post-test). E) KARPAS-422 cells were treated with TAZ and CD40L for 4 or 7 days (1 µM TAZ, 100 ng/mL CD40L and DMSO control) or in combination. CD40 ligand did not affect H3K27 tri-methylation as a single agent or in combination with TAZ.

Figure 4: Tazemetostat induces memory cell gene sets and PRDM1 (BLIMP1) expression independently of EZH2 mutation status or tazemetostat sensitivity. A) EZH2 inhibition induces a gene set that is heavily enriched for memory cell genes in DLBCL cell lines. RNAseq data from KARPAS-422 (EZH2 mutant GCB), Farage (EZH2 WT GCB), SU-DHL-5 (EZH2 WT GCB) and TMD8 (EZH2 WT ABC) treated with 1 µM TAZ for 4 days was analyzed using GSEA (Broad Institute) for enrichment of the GSE11961 memory B-cell vs. germinal center B-cell gene set (31). Normalized Enrichment Scores (NES) and False Discovery Rate scores (FDR) are indicated. B and C) KARPAS-422 (EZH2 mutant GCB), Farage (EZH2 WT GCB), SU-DHL-5 (EZH2 WT GCB) and TMD8 (EZH2 WT ABC) cell lines were treated for 7 days with 1 µM TAZ or DMSO. PRDM1 (BLIMP1) expression was evaluated by qPCR B) or western blot C) relative to DMSO control. D) CHIP-Seq was performed on cell pellets from Farage cells with or without a 1 µM tazemetostat 4-day treatment. Antibodies recognizing EZH2, H3K4me3 and H3K27me3 were used. CHIP-Seq traces from the PRDM1 locus are shown. E) SU-DHL-5 (WT EZH2) cells treated for 14 days with TAZ (6 concentrations starting at 10 µM, four-fold dilution) in combination with 100 ng/mL of CD40L leads to enhanced up-regulation of PRDM1 compared to TAZ as single agent as measured by western blot. Naive LP1 (multiple myeloma derived) cells were used as positive control for PRDM1 expression.
**Figure 1**

A) Chemical structure of a compound.

B) Bar graph showing proliferation IC50 values for different cell lines under Mutant EZH2 and Wild-Type EZH2 conditions.

C) Graphs showing cell viability over time for different cell lines with Mutant EZH2.

D) Comparison of the percentage of gated Tumor cells under different conditions.

E) Graphs showing tumor volume over time for different cell lines with TAZ treatment.
**Figure 2**

### A

**SU-DHL-5 (WT EZH2)**

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<tr>
<th>Ibrutinib (BTKi)</th>
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<td>Taz alone</td>
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### KARPAS-422 (Mut EZH2/GCB)

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### Farage (WT EZH2/GCB)

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### B

**SU-DHL-5, WT**

- 2 µM IC₅₀
- NES = -2.34
- FDR < 0.001

**KARPAS-422, mut**

- 0.0018 µM IC₅₀
- NES = -2.66
- FDR < 0.001

**Farage, WT**

- 0.099 µM IC₅₀
- NES = -1.43
- FDR < 0.001
Figure 3

A

KARPAS-422: Ibrutinib

Percent of Gated

DMSO

TAZ 1 μM

Ibrutinib 3 μM

TAZ + Ibrutinib

0

20

40

60

80

100

120

****

B

Farage: Ibrutinib

Percent of Gated

DMSO

TAZ 1 μM

Ibrutinib 3 μM

TAZ + Ibrutinib

0

20

40

60

80

100

120

**

C

SU-DHL-5: Ibrutinib

Percent of Gated

DMSO

TAZ 1 μM

Ibrutinib 3 μM

TAZ + Ibrutinib

0

20

40

60

80

100

120

****

D

KARPAS-422: CD40L

Percent of Gated

DMSO

TAZ 1 μM

CD40L 100 ng/mL

TAZ + CD40L

0

20

40

60

80

100

120

*

E

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Figure 4

A

Enrichment plot:

GSE11961_GERMALINAL_CENTRAL_BCELL_DAY7_VS_MEM
ORY_BCELL_DAY40_UP

KARPAS-422, mut
0.0018 μM IC50
NES = 1.4
PDR < 0.01

Farage, WT GCB
0.099 μM IC50
NES = 1.57
FDR < 0.001

GSE11961_GERMALINAL_CENTRAL_BCELL_DAY7_VS_MEM
ORY_BCELL_DAY40_UP

SU-DHL-5, WT GCB
2 μM IC50
NES = 1.4
FDR < 0.01

B

Fold change over DMSO

1 μM TAZ
KARPAS-422
Farage
SU-DHL-5
TMD8

C

DMSO
TAZ
DMSO
TAZ
DMSO
TAZ
DMSO
TAZ

KARPAS-422
Farage
SU-DHL-5
TMD8

D

DMSO – EZH2

DMSO – H3K4me3

DMSO – H3K27me3

TAZ – H3K27me3

E

LP1

TAZ Alone

TAZ + CD40L

μM Tazemetostat

PRDM1

B-actin

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Molecular Cancer Therapeutics

EZH2 inhibition by tazemetostat results in altered dependency on B-cell activation signaling in DLBCL

Dorothy Brach, Danielle Johnston-Blackwell, Allison Drew, et al.

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