Integrative genomic analysis of Temozolomide resistance in Diffuse Large B Cell Lymphoma

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STATEMENT OF TRANSLATIONAL RELEVANCE

Despite recent advances in the treatment for diffuse large B-cell lymphoma, there remains a largely unmet need for better treatments in relapsed and refractory disease. The alkylating agent Temozolomide (TMZ) has activity in primary central nervous system lymphoma, but its role in treatment of systemic DLBCL is yet to be explored. Using integrative gene expression and methylation analysis, we herein identify a gene expression signature associated with TMZ resistance and identify Decitabine using connectivity mapping to reverse this resistance to TMZ in DLBCL cell lines. We have then proved our hypothesis in vivo showing complete regression of tumors with the combination of Decitabine and TMZ in murine xenograft experiments. These findings support prospective clinical trials with a combination of Decitabine and TMZ in patients with DLBCL.
ABSTRACT

Purpose: Despite advances, there is an urgent need for effective therapeutics for relapsed diffuse large B cell lymphoma (DLBCL), particularly in elderly patients and primary CNS lymphoma (PCNSL). Temozolomide (TMZ), an oral DNA alkylating agent routinely used in the therapy of glioblastoma multiforme, is active in PCNSL patients but the response rates are low. The mechanisms contributing to TMZ resistance are unknown.

Experimental Design: We undertook an unbiased and genome-wide approach to understand the genomic methylation and gene expression (GE) profiling differences associated with TMZ resistance in DLBCL cell lines and identify mechanisms to overcome TMZ resistance.

Results: TMZ was cytotoxic in a subset of DLBCL cell lines, independent of MGMT promoter methylation or protein expression. Using Connectivity mapping (CMAP), we identified several compounds capable of reversing the GE signature associated with TMZ resistance. The demethylating agent Decitabine is identified by CMAP as capable of reprogramming GE to overcome TMZ resistance. Treatment with DAC led to increased expression of SMAD1, a transcription factor involved in TGFB/BMP signaling, previously shown to be epigenetically silenced in resistant DLBCL. In vitro and in vivo treatment with a combination of DAC and TMZ had greater anti-lymphoma activity than either drug alone, with complete responses in TMZ resistant DLBCL murine xenograft models.

Conclusions: Integrative genome-wide methylation and GE analysis identified novel genes associated with TMZ resistance and demonstrate potent synergy between DAC and TMZ. The evidence from cell line and murine experiments supports prospective investigation of TMZ in combination with demethylating agents in DLBCL.
INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of Non-Hodgkin’s lymphoma. Although a subset of DLBCL patients can be cured with standard adriamycin-containing combination chemotherapy, there is tremendous scope for improvement in therapeutics for relapsed DLBCL.

Temozolomide (TMZ) is an orally administered DNA alkylating agent developed in the 1980s, by the UK Cancer Research Campaign, with clinical activity in patients with brain tumors such as gliomas, astrocytomas (1), malignant melanoma (2), primary CNS lymphoma (PCNSL) (3-5), cutaneous T cell lymphoma (6), and acute myeloid leukemia (5). It is an attractive chemotherapeutic drug because it reveals good bioavailability after oral administration, good tissue penetration, and has minimal side effects. Since DLBCLs are responsive to alkylating agents and DLBCL-PCNSLs respond to TMZ, this drug may be useful in systemic DLBCLs as well. The activity of DNA repair enzyme Methyl-guanine DNA methyltransferase (MGMT) is known to be a major mechanism of resistance to this drug in human cancers, with loss of MGMT function more frequently due to promoter region methylation than to gene deletion or mutation (7). Epigenetic silencing of MGMT expression by promoter methylation of MGMT is associated with improved clinical outcomes in glioblastoma (1) and it also appears to be a useful marker for predicting survival in patients with DLBCL treated with alkylating agent–based chemotherapy (8).

In the current study, we use a systems biology approach combining integrative gene expression analysis, genomic methylation analysis and connectivity mapping to identify the genomic and epigenomic differences associated with resistance to TMZ in DLBCL, and validate 5-aza-2-deoxycytidine (Decitabine, DAC) as a potential compound to overcome TMZ resistance.
MATeRIALS AND METHODS

Cell lines, culture conditions, and drug treatment

Eleven DLBCL cell lines Farage, Karpas 422, OCI-Ly3, OCI-Ly18, OCI-Ly19 (here Ly3, Ly18, and Ly19), Pfeiffer, SUDHL-4, SUDHL-6, SUDHL-8, Toledo, and WSU-NHL were cultured in RPMI 1640 medium (Cellgro; Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 100 U/mL penicillin G, and 100 μg/mL streptomycin (Cellgro), at 37°C with humidification, and DLBCL cell lines OCI-Ly1, OCI-Ly4, OCI-Ly7, and OCI-Ly10 (here Ly1, Ly4, Ly7, and Ly10) cells were grown in Iscove’s modified Dulbecco’s medium containing 10% FBS and supplemented with antibiotics. All cell lines were kindly provided by Dr. Ari Melnick (Weill Cornell Medical College, New York, NY). Temozolomide was obtained from Merck (Whitehouse Station, NJ) and formulated at stock solutions at 50mM after dissolution in growth medium. Decitabine was purchased from Sigma Aldrich (St. Louis, MO) and formulated at 1mM. All drugs were stored at between -20 and -80°C. Cells were treated in series of eight 100 ul wells for 48 hours for viability assessment, and in 3 ml wells in triplicate, for 24 or 72 hours, to determine protein amounts and mRNA level.

Cell viability assay

Cell viability was determined by a fluorometric resazurin reduction method (CellTiter-Blue; Promega) following the manufacturer’s instructions. The number of viable cells in each treated well was calculated 48 hours after treatment. Cells (100 uL; 10^5 cells per well) were plated in 96-well plates (8 replicates per condition), with 20 uL of CellTiter-Blue Reagent (Promega) added to each well. After 1 hour of incubation with the dye, fluorescence (560Ex/590Em) was measured with the FLUOstar Omega microplate reader (BMG Lab Technologies). The number of viable cells in each treated well was calculated, based on the linear least-squares regression of the
standard curve. Cell viability in drug-treated cells was normalized to their respective untreated controls. Cell counts were confirmed on the Countess automated cell counter (Life Technologies, Carlsbad, CA) according to the manufacturer’s specifications.

DNA methylation analysis by the HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP)

Genomic DNA was isolated from cell lines with the use of a standard high-salt procedure, and the HELP assay was carried out as previously described (9), (10). The assay uses comparative isoschizomer profiling, interrogating cytosine methylation status on a genomic scale. Briefly, genomic DNA from the samples was digested by a methylcytosine-sensitive enzyme HpaII in parallel with MspI, which is resistant to DNA methylation, and then the HpaII and MspI products were amplified by ligation-mediated PCR. PCR conditions have been optimized to amplify fragments between 200 and 2000 base pair (bp), thus ensuring the preferential amplification of cytosine-phosphate-guanosine (CpG) dinucleotide-dense regions. Each fraction is then labeled with a specific dye and cohybridized onto a microarray designed to cover HpaII amplifiable fragments (HAFs) across the genome (10). The differential digestion of DNA by the 2 restriction enzymes HpaII (methylation sensitive) and MspI (methylation insensitive) assays the methylation of genomic DNA covered in the microarray probes. Detailed descriptions of HELP methods and conditions have been previously published (9), (10). DNA methylation was measured as the log (HpaII/MspI) ratio, ranging from −3.17 to 6.05, where HpaII reflects the hypomethylated fraction of the genome and MspI represents the whole genome reference. Fractions were labeled with the use of cyanine–labeled random primers (9-mers) and then hybridized onto a human HG17 custom-designed oligonucleotide array (50-mers) covering 25,626 HAFs located at gene promoters and imprinted regions. HAFs are defined as genomic sequences contained between 2 flanking HpaII sites found within 200 to 2000 bp from each other. Each HAF on the array is represented by 15 individual probes randomly distributed
across the microarray slide. All samples for microarray hybridization were processed at the Roche-NimbleGen Service Laboratory. Scanning was performed with the use of a GenePix 4000B scanner (Axon Instruments). PCR fragment length bias was corrected by quantile normalization. Further quality control and data analysis of HELP microarrays were performed as described in Thompson et al. (11). All microarray data have been submitted to the Gene Expression Omnibus repository.

**Quantitative DNA methylation analysis by MassARRAY EpiTyper**

Validation of HELP findings was performed by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry by MassARRAY (Sequenom, CA) as previously described (12). With Methprimer program, 2 primer sets (amplicons) were generated to analyze methylation of MGMT promoter. First amplicon covers exon 1 in MGMT gene and second one ~ 300 bp upstream (Figure 1B). Briefly, PCR primers specific for bisulfite-converted genomic DNA were designed using Sequenom EpiDesigner to cover the flanking HpaII sites for a given HAF, as well as any other HpaII sites found up to 2,000 bp upstream of the downstream site and up to 2,000 bp downstream of the upstream site (Primer sequences indicated in Supplementary Table S1).

**Microarray data analysis, gene network and gene ontology analysis**

Gene expression data were obtained using the Affymetrix HuGene ST 1.0 GeneChip; mRNA isolation, labeling, hybridization, and quality control were carried out as described previously (10). Raw data were processed using the Robust Multi-Averaging (RMA) algorithm and Affymetrix Expression Console software. Data are available in the NCBI Gene Expression Omnibus (GEO) database (accession number GSE27255; National Center for Biotechnology Information, Gene Expression Omnibus database. http://ncbi.nlm.nih.gov/geo). A total of 33,297 probes were measured on the array. The association between gene expression and cell line...
resistance was assessed using a conventional T-test with a p-value < 0.01 and differences of mean of genes differentially expressed between resistant and sensitive cell lines >1.0. This cutoff was chosen in order to provide a reasonably-sized set of probes and to increase the likelihood of detecting biologically significant changes in expression levels. The Database for Annotation, Visualization and Integrated Discovery (13) and Ingenuity Pathway Analysis software were used to carry out network composition analyses (http://www.ingenuity.com/products/pathways_analysis.html).

The GEP data was then analyzed with the Broad Institute’s CMAP database (http://www.broadinstitute.org/cmap/index.jsp), using the same set of differentially expressed genes in resistant vs. sensitive cell lines. With CMAP, our imported query was compared with established signatures of therapeutic compounds (or “perturbagens”). Each compound was assigned a connectivity score (from +1 to -1), representing relative association with our specific query. Compounds with connectivity scores closest to -1 were considered most likely capable of reversing the gene pattern of our query (i.e., overcoming resistance), and were therefore considered the best candidates for functional validation in an attempt to confer TMZ sensitivity.

**Quantitative Real time Polymerase Chain Reaction (RT-PCR)**

RNA was extracted from cells using TRIzol (Invitrogen, Carlsbad, CA) as in the manufacturer’s protocol. cDNA was prepared using Superscript III First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) and detected by SybrGreen (Applied Biosystems, Foster City, CA) on an Opticon2 thermal cycler (MJ Research, Waltham, MA). Gene expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT) and expressed relative to untreated control using the $\Delta\Delta CT$ method. Thermal cycler conditions were: initial step of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C (denature) and 1 min at 60°C (anneal/extend). For primers, see Supplementary Table S2.
Western blot analysis

Cells were lysed in modified RIPA buffer containing 50 mM Tris-HCl (pH=8.0), 300 mM NaCl, 10% NP-40, 1% sodium deoxycholate, and 0.1% SDS, and a protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN). Protein extracts, approximately 30 ug of each sample, were resolved by SDS-PAGE followed by immunoblotting with MGMT antibody (C-11, HRP, goat polyclonal antibody, Santa Cruz Biotechnologies, Santa Cruz, CA), phospho-SMAD1/5, SMAD1 (both Cell Signaling, Danvers, MA), and actin antibody (C-11, HRP, goat polyclonal antibody, Santa Cruz Biotechnologies, Santa Cruz, CA), and detected by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology, Santa Cruz, CA). After treatment, cells were harvested and washed with ice-cold PBS, and subsequently lysed with RIPA buffer with fresh protease and phosphatase inhibitors. Blot patterns were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/), providing a quantitative measure of protein expression.

In vivo tumor models

All animal studies were carried out in accordance with the guidelines of the Institute for Animal Studies at the Albert Einstein College of Medicine. Five million of SUDHL-6, Ly10 or Ly3 cells were mixed with 80% Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously into the right flank of four–five week-old athymic Nude-Foxn1nu female mice (Harlan Laboratories, Indianapolis, IN). When the tumors approached 0.5-0.7 cm in diameter at around 10-14 days post injection of cancer cells, the mice were divided into 4 groups: (1) TMZ alone group, which received a dose of 50mg/kg by oral gavage for a total dose 250mg/kg during 5 days on day 1, 2, 3, 4, and 5 as previously published for PCNSL models (14); (2) DAC alone group, which received 3 injections of 0.2mg/kg given by i.p. every other day for 2 weeks (day 1, 3, 5, 8, 10, and 12) - 1.2mg/kg total dose; (4) combination group, which received both TMZ and DAC at doses described above; (1) control group, which received saline by oral gavage during 5 days on day 1, 2, 3, 4, and 5. Tumors volume and weight were assessed every three days. The
data were expressed as average tumor volume (mm³) per group as a function of time. Animals were sacrificed when one-dimensional tumor diameter exceeded 2.0 cm or after loss of >10% body weight in accordance with institutional guidelines. Complete response was defined as nonpalpable tumor.

RESULTS

Temozolomide has activity in a subset of DLBCL cell lines independent of MGMT expression or promoter methylation

A panel of 15 DLBCL cell lines was exposed to different doses of TMZ (25-2500μM) in both single and multi-day schedules (50μM of TMZ every 24 hours for 5 days). We divided our panel into two groups “sensitive” and “resistant” using a single dose IC50 cutoff of 150μM, which represents the clinically achievable concentration of drug (2) (Figure 1A). To determine whether MGMT expression or promoter methylation could predict resistance or sensitivity amongst the DLBCL cells, we first compared MGMT protein and mRNA levels to TMZ response in these cell lines (Figure 1A, Supplementary Figure S1A). All TMZ-sensitive cell lines expressed some MGMT protein/mRNA, however lack of MGMT did not confer sensitivity to TMZ, as 50% of TMZ-resistant cell lines were MGMT negative.

Further, samples were analyzed for methylation of CpG sites within the MGMT gene promoter using MassARRAY. There was an inverse correlation between MGMT expression and promoter hypermethylation as shown on Figure 1B. However, there was no correlation between TMZ sensitivity (IC50 levels) and MGMT promoter methylation, as well as mRNA expression or MGMT protein expression (R value=0.0445 for mRNA) (Supplementary Figure S1A).

Gene expression and Connectivity mapping identify compounds targeting gene expression programs associated with TMZ resistant DLBCL
Gene expression data from 14 DLBCL cell lines were obtained using the Affymetrix HuGene ST 1.0 GeneChip. A total of 33,297 probes were measured on the array. Clustering gene expression profiles by Principal Component Analysis discriminated TMZ resistant and TMZ sensitive DLBCL cell line samples, indicating that the distribution of gene expression data is quite different between these groups (Figure 2A). We identified 425 significantly differentially expressed probes using cutoff at \( P<0.01 \) and \( DM>1.0 \) as shown (Figure 2B, Supplementary Table S3). To identify functional groups enriched by differentially expressed genes, these 425 probes were entered into the Database for Annotation, Visualization and Integrated Discovery (DAVID). The top functional groups of genes overexpressed in TMZ resistant cell lines involve immune response, cell activation, apoptosis, and programmed cell death (Tables 1, Supplementary Table S4). Gene Set Enrichment Analysis (GSEA) was used to categorize members of a gene set by gene families with transcription factors representing the largest gene family (Table 2).

The gene expression data was then analyzed with the Broad Institute’s CMAP database, using the same set of differentially expressed genes in TMZ resistant vs. sensitive cell lines. CMAP provided a ranked list of candidate compounds in order of likelihood of efficacy for reversing the GEP-associated with TMZ resistance. In this list, demethylating drug Decitabine (15), inhibitor of glucosylceramide synthetase DL-PPMP, antiepilepsy drug Topiramate, and inhibitor of arachidonic acid 5-lipoxygenase BW-B70C were identified within the top 10% of the candidate compounds (Figure 2C). Decitabine, a pyrimidine nucleoside analog of cytidine, was approved by the FDA as an orphan drug for treatment of myelodysplastic syndromes (16). DAC is of particular interest because epigenetic reprogramming in cancer using DAC has been shown to overcome chemotherapy resistance (17). Moreover, the specific combination of DAC and extended-schedule TMZ was capable of reversing clinical TMZ resistance in melanoma (18).
Aberrantly methylated and silenced transcription factors in TMZ resistant DLBCL can be induced by DAC

Using HELP (genome wide methylation assay), we identified candidate genes that are differentially methylated in TMZ resistant vs. sensitive cell lines (Figure 3A). We further compared differentially methylated and expressed probes in TMZ resistant DLBCL cell lines and found 152 Refseq probes corresponding to 160 genes that were both differentially methylated and expressed (Figure 3B, Supplementary Table S5). Nineteen out of 160 differentially methylated and expressed genes, i.e. C7orf54, CCNJ, CDKN2AIP, CENPJ, HNRNPD, IGLL1, LOC100287552, MAPKAPK5, METTL13, PLK4, S1PR4, SMAD1, SPTBN1, STAG2, TMSB15B, TOP2B, ZNF551, ZNF567, and ZNF74, are found to be hyper methylated and under expressed in TMZ-resistant DLBCL cell lines as shown on Figure 3C. HNRNPD, SMAD1, ZNF551, ZNF567, and ZNF74 are involved in regulation of transcription; MAPKAP5 and PLK4 are protein kinases, IGLL1 is known to be one of B-cell differentiation markers and TOP2B controls DNA replication. We are particularly interested in these genes, because their expression can be potentially induced using demethylating agent DAC (detected by CMAP as a candidate compound). TGFB/BMP signaling is known to affect proliferation, survival and differentiation of lymphocytes. Repression of SMAD1 in particular has been associated with resistance to doxorubicin in DLBCL (19). In our experiments, DAC induced SMAD1 mRNA 3-9 fold, up to 3.5 fold, and 2-fold in SUDHL-6, Ly10, and Ly3 DLBCL cells, respectively (Supplementary Figure S2B-D). We confirmed our findings by Western blotting validation of SMAD1 and phospho-SMAD1 protein expression after DAC treatment (Figure 3D, Supplementary Figure 2E). TGF-beta induction of phospho-SMAD1 was used as a positive control in these experiments. Treatment of the DLBCL cells with DAC also led to significant mRNA expression of other genes silenced in TMZ-resistant DLBCL cell lines – IGLL1 and TOP2B (Supplementary Figure S2B-D).
DAC is synergistically cytotoxic in combination with TMZ in vitro and in vivo causing complete responses in TMZ resistant DLBCL xenograft models

We next asked the question whether the combination of DAC and TMZ could overcome resistance to TMZ in DLBCL. We pre-treated TMZ resistant cell lines SUDHL-6, Ly10, and Ly3 cells with hypomethylating (i.e. non DNA damaging) doses of DAC for 72 hours and then with IC25-IC50 concentrations of TMZ for another 48 hours. We found that DAC synergizes with TMZ in reduction of cell viability (Figure 4A-B, Supplementary Figure S3A). The drug combination indices for DAC and TMZ were well below 1 cutoff for Ly3, Ly10, and SUDHL-6 cells representing the synergistic effect of the 2 drugs (Figure 4A-B, Supplementary Figure S3A).

In vivo, we used three cell lines SUDHL-6, Ly10 and Ly3 for the xenograft experiments. Mice were divided into 4 treatment cohorts: vehicle control, TMZ alone, and DAC alone and the combination of DAC and TMZ were evaluated. TMZ as a single agent had a statistically significant tumor growth inhibition compared both with control and cohort treated with DAC single drug in SUDHL-6 murine xenograft model (Figure 4C). Four out of nine animals (44.4%) had complete response by day 21 of the beginning of treatment in TMZ alone group. However, the combination of TMZ and DAC caused complete response in nine out of nine mice (100%) by day 18 after treatment started. Statistically significant tumor growth inhibition was observed in DAC alone cohort of animals comparing to control group by day 24 (P <.03) (Figure 4C). There was no significant weight loss (i.e. > 10%) in all the treated mice. For Ly10 and Ly3 murine xenograft models, five out five (100%) mice had complete response by day 9 and day 12 of the beginning of treatment in drug combination group, respectively (Figure 4D, Supplementary Figure S3B). TMZ alone treatment caused complete response in all the mice in the group by day 15 in Ly10 xenograft model and potent tumor suppression in Ly3 xenograft model (Figure 4D, Supplementary Figure S3B).
DISCUSSION

In this study, we demonstrate that TMZ has activity in a subset of DBLCL cell lines and determine the genomewide methylation and RNA expression changes associated with TMZ resistance. Using a system biology approach, we identify the demethylating agent DAC as capable of reversing the GEP associated with TMZ resistance. Remarkably, TMZ in combination with low doses of demethylating agent DAC has a significant antitumor activity achieving complete responses in TMZ resistant DLBCL murine xenograft models.

Promoter methylation has been associated with MGMT silencing and improved outcomes in glioma patients treated with TMZ (15). Favorable responses to TMZ chemotherapy have recently been reported in PCNSL patients with methylated MGMT promoters, whereas the unmethylated MGMT promoters were associated with nonresponsive recurrent PCNSL cases (17). Whether this occurs in DLBCL treated with TMZ is less clear. Prior studies examining MGMT methylation, expression and its correlation with DLBCL outcomes have conflicting results. In primary nodal DLBCLs, a good correlation was observed between the presence of MGMT expression and the unmethylated status of MGMT promoter but the absence of IHC expression was poorly correlated with the MGMT promoter methylation (20). Studies in RCHOP treated DLBCL patients have not shown a difference in outcomes according to MGMT methylation status and MGMT expression (20). Our cell line results show concordance between MGMT methylation and expression but do not correlate with sensitivity to TMZ treatment. This suggests a tumor specific association between MGMT methylation and TMZ resistance. The GCB/ABC profile of our panel of DLBCL cell lines does not seem to correlate with TMZ sensitivity either (Figure 1A). We therefore undertook a genome-wide search for the determinants of TMZ resistance and mechanisms to overcome them.
Connectivity mapping can identify biomarkers for predicting response to drugs, mechanisms of resistance, and ways to overcome them (21-23). One limitation of CMAP may be the limited drugs or genesets included in the database. In our study CMAP identified several potential agents to overcome TMZ resistance in DLBCL. Reversal of promoter DNA hypermethylation and associated gene silencing is an attractive cancer therapy approach. DAC and its analog azacitidine, AZA (24), two major DNA demethylating agents, have recently emerged as potent therapies for the preleukemic hematological diseases (17). At high doses, DAC was reported to induce rapid DNA damage and cytotoxicity, while clinically relevant nanomolar doses of DAC can reprogram stem cells to lead to sustained remission in xenograft models treated at low doses (17). Our results showing efficacy of combination treatment may be relevant to elderly patients, especially with the advent of oral AZA.

Recent epigenomic studies in lymphomas and leukemias suggest that measurement of genome-wide DNA methylation patterning may be more informative than gene expression profiling alone, and can identify biologically and clinically important patient sub-populations (25), (26). Knowing the methylation status of the genes can be useful to narrow down differentially expressed gene lists to select therapeutically relevant candidates (25, 27). We used integrative epigenomic analysis to identify SMAD1 as a therapeutic target in TMZ-resistant DLBCL. SMAD proteins, the mammalian homologs of the Drosophila Mothers against dpp (Mad) are major signaling molecules acting downstream of TGFβ/BMP signaling pathway (24, 28). SMAD1 (also designated Madr1 or JV4-1), SMAD5 and mammalian SMAD8 are effectors of BMP2 and BMP4 function while SMAD2 and SMAD3 are involved in TGFβ and activin-mediated growth modulation (24, 29). Following BMP or TGFβ binding to the targeted surface receptors, SMAD1 becomes phosphorylated at Ser-463 and Ser-465. Activated SMAD1 associates with SMAD4 and translocates to the nucleus, where the SMAD1-SMAD4 complex interacts with the transcriptional coactivators CREB-binding protein, p300 and others to
stimulate SMAD1-dependent transcription (28, 29). Although SMAD1 and SMAD5 primarily relay signals initiated by the BMP family of cytokines, they are also phosphorylated by TGFβ1 in DLBCL (29). Conditional knockout of SMAD1/5 in mice leads to metastatic granulosa cell tumor development, implicating a strong role of SMAD1/5 as critical tumor suppressors (30). Our in vitro results suggest that SMAD1 expression may contribute to TMZ sensitization in DLBCL. Based on recent investigation, SMAD1 hypermethylation in patients with lymphoma was associated with more chemoresistant subtypes of DLBCLs (ABC-DLBCLs) and poorer overall survival after standard therapy (19). Prolonged exposure to low-dose DNMT inhibitors (DNMTI) reprogrammed chemoresistant cells to become doxorubicin sensitive while reactivation of SMAD1 was required for chemosensitization (19). The other 18 genes hyper-methylated in TMZ resistant DLBCL cell lines may also serve as potential biomarkers for response to TMZ.

In summary, our results demonstrate that the integration of genome-wide promoter methylation, gene expression, and drug discovery using connectivity mapping can be effective in identification of novel therapeutic targets and mechanisms of overcoming drug resistance. We also identify demethylating agent DAC treatment as capable of producing complete responses in vivo in TMZ-resistant DLBCL and suggest epigenetic repression of TGFβ/SMAD1 as a resistance mechanism, which can be potently overcome in vivo using DAC to improve the depth of response in patients with relapsed DLBCL or PCNSL.

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**Note:**

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References


Table 1. Functional groups enriched by DAVID gene ontology analysis of overexpressed genes in TMZ resistant DLBCL cell lines.

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Table 2. GSEA categorizes differentially expressed in TMZ resistant cell lines by gene families.

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</tbody>
</table>
Figure Legends

Figure 1: MGMT expression and MGMT promoter methylation is a poor predictor of sensitivity to TMZ in DLBCL.

A. $10^6$ DLBCL cells/ml was treated with TMZ, 5-2500μM for 48h. DLBCL cell lines corresponding to the dose/response curves are labeled in order of degree of resistance. Red line separates two groups of DLBCL cell lines based on resistance to TMZ. Green line represents plasma TMZ concentrations in patients with metastatic malignant melanoma treated orally with 200 mg/m² of the drug. Data represents average of 8 replicates per condition in two independent experiments. MGMT protein expression in DLBCL detected by Western blotting analysis is shown below. B. MGMT promoter methylation correlates inversely with MGMT expression in DLBCL. Scale shows percentage of methylation from 0% (low, in red) to 100% (high, in yellow) for each CpGs.

Figure 2: Gene expression, Connectivity mapping analysis, and profiling of aberrantly methylated genes identify compounds targeting set of genes overexpressed in TMZ resistant DLBCL.

A. Three-dimensional principal component analysis of Gene expression data comparing TMZ resistant DLBCL cell lines (red) and TMZ sensitive DLBCL cells (blue). B. Heat map demonstrating the signature of 425 significantly differentially expressed genes (P<0.01, DM>1.0). C. Connectivity mapping output identifies demethylating agent Decitabine within the top 10% of perturbagens. A high negative connectivity score indicates that the corresponding perturbagen reversed the expression of the query signature.

Figure 3: Aberrantly methylated and expressed genes in TMZ resistant DLBCL can be targeted by DAC:
A. Volcano plot of methylation differences of mean between TMZ resistant and sensitive DLBCL (X axis) vs. significance (Y axis). B. A Venn diagram of the overlap in differentially expressed loci (p<0.05, DM>0.5) and differentially methylated loci (p<0.05, DM>0.5) between TMZ Resistant and Sensitive DLBCL. C. A Venn diagram of the overlap in under expressed genes (p<0.05, DM>0.5) and hyper methylated genes (p<0.05, DM>0.5) between TMZ Resistant and Sensitive DLBCL. Overlapped gene list is shown in a black box. D. DAC treatment leads to increased expression of phosphorylated and total level of transcriptional factor SMAD1. Western blotting analysis shows phospho-SMAD1 and SMAD1 protein expression after treatment of SUDHL-6 and Ly10 DLBCL cells with 0.1 μM of DAC every 24hrs for 3 days. Treatment of cells with TGFβ1 at 10ng/ml for 1 hr was used as a positive control. The ratio of SMAD1 to actin and ph-SMAD1/5 to actin was determined by densitometry using ImageJ.

Figure 4: DAC synergizes with TMZ in inhibition of tumor growth in vitro and in vivo causing complete response in TMZ resistant DLBCL xenograft models.

A, B. DAC sensitizes chemo-refractory DLBCL cells to TMZ in vitro in SUDHL-6 and Ly10 cells, respectively. Combination indices (CI) values were determined using the Chou-Talalay equation, as calculated by CalcuSyn software. C, D. TMZ and DAC synergize in SUDHL-6 and Ly10 DLBCL xenograft models. Plots represent summary of two independent experiments for each cell line. N=7 in DAC alone and control groups; N=9 in TMZ alone and combination groups for SUDHL-6 xenograft experiments. N=5 in each group for Ly10 DLBCL xenograft model.
Figure 1
Figure 2

A. Principal Component Analysis

B. Resistant vs. Sensitive

C. Heatmap with Cmap score and permutation name:

<table>
<thead>
<tr>
<th>Cmap name</th>
<th>Cmap score</th>
<th>Permutant name</th>
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<tbody>
<tr>
<td>DL-PPMP</td>
<td>-0.708</td>
<td>Inhibitor of glucosylceramide synthetase</td>
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<tr>
<td>topiramate</td>
<td>-0.679</td>
<td>Antiepilepsy drug</td>
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<tr>
<td>BW-B70C</td>
<td>-0.610</td>
<td>Inhibitor of arachidonic acid 5-lipoxygenase</td>
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<td>rituximab</td>
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<td>Platelet-derived growth factor</td>
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<tr>
<td>phenyl biguanide</td>
<td>-0.533</td>
<td>5-HT3 receptor agonist</td>
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Leshchenko et al.
Figure 4

A. SUDHL-6

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B. Ly10

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<td>0.5/1500</td>
</tr>
<tr>
<td>2</td>
<td>1/750</td>
</tr>
</tbody>
</table>

C. SUDHL-6

D. Ly10

control
DAC
TMZ
TMZ+DAC
Clinical Cancer Research

Integrative genomic analysis of Temozolomide resistance in Diffuse Large B Cell Lymphoma

Violetta Leshchenko, Pei-Yu Kuo, Zewei Jiang, et al.

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