Cancer Therapy: Preclinical

Integrative Genomic Analysis of Temozolomide Resistance in Diffuse Large B-Cell Lymphoma

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Abstract

Purpose: Despite advances, there is an urgent need for effective therapeutics for relapsed diffuse large B-cell lymphoma, particularly in elderly patients and primary central nervous system (CNS) lymphoma. Temozolomide (TMZ), an oral DNA-alkylating agent routinely used in the therapy of glioblastoma multiforme, is active in patients with primary CNS lymphoma 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 profiling differences associated with TMZ resistance in diffuse large B-cell lymphoma cell lines and identify mechanisms to overcome TMZ resistance.

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

Conclusions: Integrative genome-wide methylation and gene expression 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 diffuse large B-cell lymphoma.

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Introduction

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

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 central nervous system (CNS) lymphoma (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. Because diffuse large B-cell lymphomas are responsive to alkylating agents and diffuse large B-cell lymphoma–primary CNS lymphomas respond to TMZ, this drug may be useful in systemic diffuse large B-cell lymphomas 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...
diffuse large B-cell lymphoma 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 Map (CMAP) to identify the genomic and epigenomic differences associated with resistance to TMZ in diffuse large B-cell lymphoma, 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 diffuse large B-cell lymphoma 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 RPMI1640 medium (Cellgro) supplemented with 10% FBS (Gemini Bio-Products), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 100 IU/mL penicillin G, and 100 μg/mL streptomycin (Cellgro), at 37°C with humidification, and diffuse large B-cell lymphoma cell lines. We have then proved our hypothesis in vivo showing complete regression of tumors with the combination of DAC and TMZ in murine xenograft experiments. These findings support prospective clinical trials with a combination of DAC and TMZ in patients with diffuse large B-cell lymphoma.

DNA methylation analysis by the HpaII tiny fragment enrichment by ligation-mediated PCR
Genomic DNA was isolated from cell lines with the use of a standard high-salt procedure, and the HpaII tiny fragment enrichment by ligation-mediated PCR (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 withMspI, 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 2,000 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 cobybridized onto a microarray designed to cover HpaII amplifiable fragments (HAF) across the genome (10). The differential digestion of DNA by the two 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 two flanking HpaII sites found within 200 to 2,000 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 by Thompson and colleagues (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) as previously described (12). With the Methprimer program, two primer sets (amplicons) were designed to analyze methylation of MGMT promoter. The first amplicon covers exon 1 in MGMT gene and the second is approximately 300 bp upstream (Fig. 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 value of less than 0.01 and differences of mean of genes differentially expressed between resistant and sensitive cell lines that were greater than 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 gene expression profiling (GEP) data were 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 versus 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 a relative association with our specific query. Compounds with connectivity scores closest to −1 were considered most likely to be 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**

RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was prepared using Superscript III First Strand cDNA synthesis kit (Invitrogen) and detected by SybrGreen (Applied Biosystems) on an Opticon2 thermal cycler (MJ Research). Gene expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT) and expressed relative to untreated control using the 2^ΔΔCT method. Thermal cycler conditions were: initial step of 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C (denature) and 1 minute at 60°C (anneal/extend). For primers, see Supplementary Table S2.

**Western blot analysis**

Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mmol/L Tris–HCl (pH = 8.0), 300 mmol/L NaCl, 10% NP-40, 1% sodium deoxycholate, and 0.1% SDS, and a protease inhibitor cocktail tablet (Roche Applied Science). Protein extracts, approximately 30 μg of each sample, were resolved by SDS-PAGE followed by immunoblotting with MGMT antibody [C-20, goat polyclonal antibody; Santa Cruz Biotechnology], phospho-SMAD1/5, SMAD1 (both from Cell Signaling Technology), and actin antibody (C-11, horseradish peroxidase (HRP), goat polyclonal antibody; Santa Cruz Biotechnology), and detected by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology). 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 50% Matrigel (BD Biosciences) and injected subcutaneously into the right flank of each animal. Tumor size was measured weekly and recorded as the product of the maximum tumor diameter (mm) and the diameter perpendicular to it (mm). The volume of each tumor was calculated as: (width x length x depth) / 2. The tumor volume was calculated using the following formula: Volume = 4/3 * π * (radius)^3. The drug was administered daily for 14 days starting on day 15. A F football was used to place SUDHL-6 cells into the right flank of the mice on day 0. Corresponding doses of TMZ were given on days 15, 16, 17, and 18.umor size was measured weekly and recorded as the product of the maximum tumor diameter (mm) and the diameter perpendicular to it (mm). The volume of each tumor was calculated as: (width x length x depth) / 2. The tumor volume was calculated using the following formula: Volume = 4/3 * π * (radius)^3. The drug was administered daily for 14 days starting on day 15. A F football was used to place SUDHL-6 cells into the right flank of the mice on day 0. Corresponding doses of TMZ were given on days 15, 16, 17, and 18.
flank of 4- to 5-week-old athymic Nude-Foxn1nu female mice (Harlan Laboratories, Indianapolis, IN). When the tumors approached 0.5 to 0.7 cm in diameter at approximately 10 to 14 days postinjection of cancer cells, the mice were divided into 4 groups: (i) TMZ-alone group, which received a dose of 50 mg/kg by oral gavage for a total dose of 250 mg/kg during 5 days on days 1 to 5 as previously published for primary CNS lymphoma models (14); (ii) DAC-alone group, which received three injections of 0.2 mg/kg given intraperitoneally (i.p.) every other day for 2 weeks (days 1, 3, 5, 8, 10, and 12)—1.2 mg/kg total dose; (iii) combination group, which received both TMZ and DAC at doses described earlier; (iv) control group, which received saline by oral gavage during 5 days on days 1 to 5. Tumor volume and weight were assessed every 3 days. The data were expressed as average tumor volume (mm\(^3\)) per group as a function of time. Animals were sacrificed when one-dimensional tumor diameter exceeded 2.0 cm or after loss of greater than 10% body weight in accordance with institutional guidelines. Complete response was defined as nonpalpable tumor.

Results

**TMZ has activity in a subset of diffuse large B-cell lymphoma cell lines independent of MGMT expression or promoter methylation**

A panel of 15 diffuse large B-cell lymphoma cell lines was exposed to different doses of TMZ (25–2,500 \(\mu\)mol/L) in both single and multiday schedules (50 \(\mu\)mol/L of TMZ every 24 hours for 5 days). We divided our panel into two groups, *sensitive* and *resistant*, using a single-dose IC\(_{50}\) cutoff of 150 \(\mu\)mol/L, which represents the clinically achievable concentration of drug (Fig. 1A; ref. 2). To determine whether MGMT expression or promoter methylation could predict resistance or sensitivity among the diffuse large B-cell lymphoma cells, we first compared MGMT protein and mRNA levels to TMZ response in these cell lines (Fig. 1A and Supplementary Fig. 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.

Furthermore, 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 Fig. 1B. However, there was no correlation between TMZ sensitivity (IC\(_{50}\) levels) and MGMT promoter methylation, as well as mRNA expression or MGMT protein expression (R value = 0.0445 for mRNA; Supplementary Fig. S1A).

**Gene expression and CMAP identify compounds targeting gene expression programs associated with TMZ-resistant diffuse large B-cell lymphoma**

Gene expression data from 14 diffuse large B-cell lymphoma 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 -sensitive diffuse large B-cell lymphoma cell line samples, indicating that the distribution of gene expression data is quite different between these groups (Fig. 2A). We identified 425 significantly differentially expressed probes using cutoff at \(P\) values less than 0.01 and differentially methylated (DM) greater than 1.0 as shown (Fig. 2B and 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 (Table 1 and 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 were then analyzed with the Broad Institute’s CMAP database, using the same set of differentially expressed genes in TMZ-resistant versus -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, the demethylating drug DAC (15), an 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 (Fig. 2C). DAC, a pyrimidine nucleoside analog of cytidine, was approved by the U.S. Food and Drug Administration Agency (FDA) as an orphan drug for the 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 diffuse large B-cell lymphoma can be induced by DAC**

Using HELP (genome-wide methylation assay), we identified candidate genes that are differentially methylated in TMZ-resistant versus -sensitive cell lines (Fig. 3A). We further compared differentially methylated and expressed probes in TMZ-resistant diffuse large B-cell lymphoma cell lines and found 152 Rseq probes corresponding to 160 genes that were both differentially methylated and expressed (Fig. 3B and Supplementary Table S5). Nineteen out of 160 differentially methylated and expressed genes, that is, C7orf54, CCNJ, CDKN2AIP, CENPJ, HNRNPD, IGLL1, LOC100287552, MAPKAPK5, METTL13, PLK4, S1PR4, SMAD1, SPTBN1, STAG2, TBMB15B, TOP2B, ZNF551, ZNF567, and ZNF74, are found to be hypermethylated and under expressed in TMZ-resistant diffuse large B-cell lymphoma cell lines as shown on Fig. 3C. HNRNPD, SMAD1, ZNF551, ZNF567, and ZNF74 are involved in the regulation of transcription; MAPKAPK5 and PLK4 are protein kinases; IGLL1 is known to be one of the B-cell regulative transcription factors.
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). TGF-β/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 diffuse large B-cell lymphoma (19). In our experiments, DAC induced SMAD1 mRNA 3- to 9-fold, up to 3.5-fold, and 2-fold in SUDHL-6, Ly10, and Ly3 diffuse large B-cell lymphoma cells, respectively (Supplementary Fig. S2B–D). We confirmed our findings by Western blot validation of SMAD1 and phospho-SMAD1 protein expression after DAC treatment (Fig. 3D and Supplementary Fig. S2E). TGF-β induction of phospho-SMAD1 was used as a positive control in these experiments. Treatment of the diffuse large B-cell lymphoma cells with DAC also led to...
TMZ-resistant diffuse large B-cell lymphoma cell lines—
IGLL1 and TOP2B (Supplementary Fig. S2B–D).

DAC is synergistically cytotoxic in combination with
TMZ in vitro and in vivo causing complete responses in
TMZ-resistant diffuse large B-cell lymphoma xenograft
together with IC25 to IC50 concentrations of TMZ for another 48
hours. We found that DAC synergizes with TMZ in reduc-
tion of cell viability (Fig. 4A–4B and Supplementary Fig.
S3A). The drug combination indices for DAC and TMZ were
determined. 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 (Fig. 4C). Four out of nine animals
(44.4%) had complete response by day 21 after beginning
treatment in the TMZ-alone group. However, the combi-
nation 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 the DAC-alone cohort of animals compared with
control group by day 24 (P < 0.03; Fig. 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 the drug combination group,
respectively (Fig. 4D and Supplementary Fig. 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 (Fig. 4D and
Supplementary Fig. 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 systems biology approach, we
identify the demethylating agent DAC as being capable of
reversing the GEP associated with TMZ resistance. Remark-
ably, TMZ in combination with low doses of demethylating
agent DAC has a significant antitumor activity, achieving
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Promoter methylation has been associated with MGMT
silencing and improved outcomes in patients with glioma
patients with TMZ (15). Favorable responses to TMZ che-
motherapy have recently been reported in patients with
primary CNS lymphoma with methylated MGMT promot-
ers, whereas the unmethylated MGMT promoters were
associated with cases of nonresponsive recurrent primary
tumor suppression in Ly3 xenograft model (Fig. 4D and
Supplementary Fig. S3B).

Table 1. Functional groups enriched by DAVID gene ontogenic analysis of overexpressed genes in
TMZ-resistant diffuse large B-cell lymphoma cell lines.

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Count</th>
<th>P value Bonferroni</th>
<th>Benjamini</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006955–immune response</td>
<td>29</td>
<td>5.29E–10</td>
<td>6.48E–07</td>
<td>8.57E–07</td>
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<td>GO:0051222–positive regulation of protein transport</td>
<td>8</td>
<td>6.22E–06</td>
<td>0.00756225</td>
<td>0.00380451</td>
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<tr>
<td>GO:0001775–cell activation</td>
<td>13</td>
<td>4.94E–05</td>
<td>0.05875402</td>
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<td>GO:0006915–apoptosis</td>
<td>18</td>
<td>1.90E–04</td>
<td>0.20762053</td>
<td>0.056518686</td>
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<td>GO:0051223–regulation of protein transport</td>
<td>8</td>
<td>1.96E–04</td>
<td>0.21301719</td>
<td>0.046780365</td>
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<td>GO:0012501–programmed cell death</td>
<td>18</td>
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<td>GO:0070201–regulation of establishment of protein localization</td>
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<td>GO:0008219–cell death</td>
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<td>GO:0016265–death</td>
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<td>GO:0051050–positive regulation of transport</td>
<td>10</td>
<td>5.74E–04</td>
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significant mRNA expression of other genes silenced in
TMZ-resistant diffuse large B-cell lymphoma cell lines—
IGLL1 and TOP2B (Supplementary Fig. S2B–D).

The drug combination indices for DAC and TMZ were
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ers, whereas the unmethylated MGMT promoters were
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Supplementary Fig. S3B).

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

<table>
<thead>
<tr>
<th>Gene family</th>
<th>Number of genes</th>
</tr>
</thead>
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<tr>
<td>Cytokines and growth factors</td>
<td>12</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>36</td>
</tr>
<tr>
<td>Homeodomain proteins</td>
<td>3</td>
</tr>
<tr>
<td>Cell differentiation markers</td>
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<td>Protein kinases</td>
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</tr>
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<td>Translocated cancer genes</td>
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<td>Oncogenes</td>
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<td>Tumor suppressors</td>
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CNS lymphoma (17). Whether this occurs in diffuse large B-cell lymphoma treated with TMZ is less clear. Prior studies examining MGMT methylation, expression, and its correlation with diffuse large B-cell lymphoma outcomes have conflicting results. In primary nodal diffuse large B-cell lymphomas, a good correlation was observed between the presence of MGMT and the unmethylated status of the MGMT promoter, but the absence of immunohistochemical (IHC) expression was poorly correlated with the MGMT promoter methylation (20). Studies in rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (RCHOP)-treated patients of diffuse large B-cell lymphoma 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 diffuse large B-cell lymphoma cell lines does not seem to correlate with TMZ sensitivity either (Fig. 1A). We, therefore, undertook a genome-wide search for the determinants of TMZ resistance and mechanisms to overcome them.

CMAP 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 gene sets included in the database. In our study CMAP identified several potential agents to overcome TMZ resistance in diffuse large B-cell lymphoma. 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-demethylation agents, have recently emerged as potent therapies for preleukemic hematologic diseases (17). At high doses, DAC was reported to induce rapid DNA damage and cytotoxicity, whereas 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 subpopulations (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 diffuse large B-cell lymphoma. SMAD proteins, the mammalian homologs of the Drosophila Mothers against dpp (Mad) are major signaling molecules acting downstream of TGF-β/BMP signaling pathway (24, 28). SMAD1 (also designated Madr1 or JV4-1), SMAD5, and mammalian SMAD8 are effectors of BMP2 and BMP4 function whereas SMAD2 and SMAD3 are involved in TGF-β and actin-mediated growth modulation (24, 29). Following BMP or TGF-β 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 SMAD3 primarily relay signals initiated by the BMP family of cytokines, they are also phosphorylated by TGF-β1 in diffuse large B-cell lymphoma (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 diffuse large B-cell lymphoma. Based on recent investigation, SMAD1

Figure 4. DAC synergizes with TMZ in inhibition of tumor growth in vitro and in vivo causing complete response in TMZ-resistant diffuse large B-cell lymphoma xenograft models. A and B, DAC sensitizes chemorefractory diffuse large B-cell lymphoma cells to TMZ in vitro in SUDHL-6 and Ly10 cells, respectively. Values of combination indices (CI) were determined using the Chou–Talalay equation, as calculated by CalcuSyn software. C and D, TMZ and DAC synergize in SUDHL-6 and Ly10 diffuse large B-cell lymphoma 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 diffuse large B-cell lymphoma xenograft model.
Genomic Analysis of Temozolomide Resistance in Diffuse Large B-Cell Lymphoma

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References


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