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) lymphomas. 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. Clin Cancer Res; 20(2); 1–11. ©2013 AACR.

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...
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 CNS lymphoma, but its role in treatment of systemic diffuse large B-cell lymphoma 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 DAC using CMAP to reverse this resistance to TMZ in 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.

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 U/mL penicillin G, and 100 μg/mL streptomycin (Cellgro), at 37°C with humidification, and diffuse large B-cell lymphoma 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. A. Melnick (Weill Cornell Medical College, New York, NY). TMZ was obtained from Merck and formulated at stock solutions at 50 mmol/L after dissolution in growth medium. DAC was purchased from Sigma Aldrich and formulated at 1 mmol/L. All drugs were stored between −20°C and −80°C. Cells were treated in series of eight 100 μL 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) according to the manufacturer’s instructions. The number of viable cells in each treated well was calculated 48 hours after treatment. Cells (100 μL; 10^5 cells per well) were plated in 96-well plates (8 replicates per condition), with 20 μL of CellTiter-Blue Reagent (Promega) added to each well. After 1 hour of incubation with the dye, fluorescence (560Ex/590Em) was measured with the FLIIOstar 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) according to the manufacturer’s specifications.

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 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 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 cohybridized 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 generated 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 EpipRinger 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 analyzed by Ingenuity Information, Gene Expression Omnibus database. A connectivity score (from 0 to 10) was assigned to each compound when comparing biologically significant changes in expression levels grouped under the same term. 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, Santa Cruz Biotechnology), phospho-SMAD1/5, SMAD1 (both from Cell Signaling Technology), and actin antibody (C-11, 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 five to eight mice per treatment group. Mice were randomized within treatment groups and were observed for tumor growth or signs of toxicity for 35 days. The experiment was terminated when the tumor volume reached a maximum of 1,000 mm^3 or when a tumour became necrotic. Tumour volume was measured using calipers and calculated using the formula: Volume = length × width ^2/2.

**Figure 1.** MGMT expression and MGMT promoter methylation are a poor predictor of sensitivity to TMZ in diffuse large B-cell lymphoma. A, diffuse large B-cell lymphoma 10^6 cells/mL were treated with TMZ, 25 to 2,500 μmol/L for 48 hours. Diffuse large B-cell lymphoma cell lines corresponding to the dose-response curves are labeled in order of degree of resistance. The red line separates two groups of diffuse large B-cell lymphoma cell lines based on resistance to TMZ. Green line represents plasma TMZ concentrations in patients with metastatic malignant melanoma treated orally with 200 mg/m^2 of the drug. Data represent average of eight replicates per condition in two independent experiments. MGMT protein expression in diffuse large B-cell lymphoma detected by Western blotting analysis is shown below. B, MGMT promoter methylation correlates inversely with MGMT expression in diffuse large B-cell lymphoma.

Scale shows percentage of methylation from 0% (low, in red) to 100% (high, in yellow) for each CpG.
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 μmol/L) in both single and multiday schedules (50 μmol/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 μmol/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 ($R = 0.0445$ 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 Refseq 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, CCNI, CDKN2AIP, CENPI, HNRNPD, IGLL1, LOC100287552, MAPKAPK5, METTL13, PLK4, S1PR4, SMAD1, SPTBN1, STAG2, TMSB15B, 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
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...
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. Remarkably, TMZ in combination with low doses of demethylating agent DAC has a significant antitumor activity, achieving complete responses in TMZ-resistant diffuse large B-cell lymphoma murine xenograft models. Promoter methylation has been associated with MGMT silencing and improved outcomes in patients with glioma patients with TMZ (15). Favorable responses to TMZ chemotherapy have recently been reported in patients with primary CNS lymphoma with methylated MGMT promoters, whereas the unmethylated MGMT promoters were associated with cases of nonresponsive recurrent primary lymphoma.

**Discussion**

Table 2. Functional groups enriched by DAVID gene ontogenic analysis of overexpressed genes in TMZ-resistant diffuse large B-cell lymphoma cell lines.

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<tr>
<th>GO Term</th>
<th>Count</th>
<th>P value</th>
<th>Bonferroni</th>
<th>Benjamini</th>
<th>FDR</th>
</tr>
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<tr>
<td>GO:0006955→immune response</td>
<td>29</td>
<td>5.29E-10</td>
<td>6.48E-07</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>
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<td>GO:0001775→cell activation</td>
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<td>GO:0006915→apoptosis</td>
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<td>GO:0051223→regulation of protein transport</td>
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<td>1.96E-04</td>
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<td>GO:0012501→programmed cell death</td>
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<td>GO:0070201→regulation of establishment of protein localization</td>
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*Table 1. Functional groups enriched by DAVID gene ontogenic analysis of overexpressed genes in TMZ-resistant diffuse large B-cell lymphoma cell lines.*

**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>
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<tr>
<td>Cytokines and growth factors</td>
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</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>
<td>15</td>
</tr>
<tr>
<td>Protein kinases</td>
<td>20</td>
</tr>
<tr>
<td>Translocated cancer genes</td>
<td>11</td>
</tr>
<tr>
<td>Oncogenes</td>
<td>12</td>
</tr>
<tr>
<td>Tumor suppressors</td>
<td>3</td>
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</tbody>
</table>

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 models

We next asked the question whether the combination of DAC and TMZ could overcome resistance to TMZ in diffuse large B-cell lymphoma. We pretreated 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 IC_{25} to IC_{50} concentrations of TMZ for another 48 hours. We found that DAC synergizes with TMZ in reduction of cell viability (Fig. 4A–4B and Supplementary Fig. S3A). The drug combination indices for DAC and TMZ were well below 1, the cutoff for Ly3, Ly10, and SUDHL-6 cells representing the synergistic effect of the two drugs (Fig. 4A–4B and Supplementary Fig. S3A).

In vitro, we used three cell lines—SUDHL-6, Ly10, and Ly3—for the xenograft experiments. Mice were divided into four treatment cohorts: vehicle control, TMZ alone, 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 (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 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 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 vivo, we used three cell lines—SUDHL-6, Ly10, and Ly3—for the xenograft experiments. Mice were divided into four treatment cohorts: vehicle control, TMZ alone, 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 (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 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 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).
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 expression 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-demethylating 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).
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 SMAD5 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 chemoresistant diffuse large B-cell lymphoma cells to TZM 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.
hypermethylation in patients with lymphoma was associated with more chemoresistant subtypes of diffuse large B-cell lymphomas (ABC-DLBCL) and poorer overall survival after standard therapy (19). Prolonged exposure to low-dose DNMT inhibitors reprogrammed chemoresistant cells to become doxorubicin sensitive whereas reactivation of SMAD1 was required for chemosensitization (19). The other 18 genes hypermethylated in TMZ-resistant diffuse large B-cell lymphoma 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 CMAP can be effective in identification of novel therapeutic targets and mechanisms of overcoming drug resistance. In addition, we identify treatment with the demethylating agent DAC as being capable of producing complete responses in vivo in TMZ-resistant diffuse large B-cell lymphoma and suggest epigenetic repression of TGF-β/SMAD1 as a resistance mechanism, which can be potently overcome in vivo using DAC to improve the depth of response in patients with relapsed diffuse large B-cell lymphoma or primary CNS lymphoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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