Mechanism-Based Epigenetic Chemosensitization Therapy of Diffuse Large B-Cell Lymphoma

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ABSTRACT

Although aberrant DNA methylation patterning is a hallmark of cancer, the relevance of targeting DNA methyltransferases (DNMT) remains unclear for most tumors. In diffuse large B-cell lymphoma (DLBCL) we observed that chemoresistance is associated with aberrant DNA methylation programming. Prolonged exposure to low-dose DNMT inhibitors (DNMTI) reprogrammed chemoresistant cells to become doxorubicin sensitive without major toxicity in vivo. Nine genes were recurrently hypermethylated in chemoresistant DLBCL. Of these, SMAD1 was a critical contributor, and reactivation was required for chemosensitization. A phase I clinical study was conducted evaluating azacitidine priming followed by standard chemoimmunotherapy in high-risk patients newly diagnosed with DLBCL. The combination was well tolerated and yielded a high rate of complete remission. Pre- and post-azacitidine treatment biopsies confirmed SMAD1 demethylation and chemosensitization, delineating a personalized strategy for the clinical use of DNMTIs.

SIGNIFICANCE: The problem of chemoresistant DLBCL remains the most urgent challenge in the clinical management of patients with this disease. We describe a mechanism-based approach toward the rational translation of DNMTIs for the treatment of high-risk DLBCL. Cancer Discov; 3(9); 1002–19. © 2013 AACR.

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INTRODUCTION

DNA methylation patterning contains epigenetic information that encodes the transcriptional programming and phenotype of normal and malignant cells (1). Aberrant DNA hypermethylation of tumor suppressor genes can result in their inappropriate transcriptional silencing and thus contribute to loss of checkpoints and other functions in cancer. For example, CpG methylation suppresses the promoter and reduces the expression of the tumor suppressor gene CDKN2A in non-Hodgkin lymphomas (NHL; ref. 2), an event associated with more aggressive variants of the disease (3). Inactivation of tumor suppressor pathways is an important contributor of resistance to chemotherapy in cancer (4–6), in part because the activity of most chemotherapy agents depends to a great extent on the same proapoptotic and prodifferentiation pathways that are disabled during carcinogenesis. Inactivation of these pathways by mutations or hypermethylation can therefore affect drug sensitivity (4, 7). Gene-specific and genomic alterations in DNA methylation have been described in the various subtypes of NHL (8–14). Moreover, integrated DNA methylation and gene expression profiling identified specific methylation signatures in the activated B cell (ABC) and germinal center B cell (GCB) subtypes of diffuse large B-cell lymphomas (DLBCL), suggesting that these are epigenetically distinct entities (12).

CpG dinucleotides are methylated by DNA methyltransferase (DNMT1), DNMT3A, and DNMT3B. DNMT1 is predominantly involved in maintaining, whereas DNMT3A and DNMT3B primarily mediate de novo cytosine methylation. Inhibition of DNMT activity can reverse DNA methylation and gene silencing and therefore restore expression of important gene pathways (1). 5-aza-2′-deoxycytidine and azacitidine are pyrimidine nucleoside analogs of cytosine that incorporate into DNA and irreversibly inactivate DNMTs by forming a covalent bond between the 5-azacytosine ring and the enzyme (15). As a consequence, DNMTs become unable to efficiently introduce methyl groups in newly synthesized DNA strands, resulting in the gradual depletion of 5-methylcytosines from the genome as cells divide. These studies raise the possibility that DNMT inhibitors (DNMTI) might be useful in tumors with active DNA replication. In this regard, tumors with high proliferative ratios like DLBCL (16) might be susceptible to these agents.

Patients with DLBCL treated with current standard therapy, generally consisting of rituximab administered with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP), obtain complete response rates of approximately 75% with long-term disease-free survival of approximately 60% (17). The International Prognostic Index (IPI) defines risk groups based on clinical factors at presentation, including age, stage, performance status, multiple extranodal sites,
and lactate dehydrogenase levels (18). Patients with multiple risk factors have a significantly poorer outcome than average. In a minority of patients whose lymphomas recur after initial therapy, second-line therapy followed by high-dose chemotherapy and autologous stem cell transplant provides a second chance for cure. However, many patients will not respond to aggressive second-line treatments due to refractory disease (17). In addition, a significant number of patients may have difficulty tolerating intensive second-line therapy due to age and/or comorbidities. Despite the improvements in overall survival of patients with DLBCL with the routine addition of rituximab therapy, approximately one third of patients have disease that is either refractory or relapses after initial therapy. The fact that the majority of these patients will die within two years of diagnosis underlines the need for new therapeutic approaches to improve long-term outcomes.

Taking together (i) the occurrence of aberrant DNA methylation patterning in DLBCL, (ii) the possibility that aberrant DNA methylation might contribute to the lymphoma phenotype and repress genes that play a role in chemoresponsiveness, and (iii) the high proliferative rate of DLBCL cells which could facilitate the mechanism of action of DNMTIs, we hypothesized that DNMTIs will be therapeutically active in this disease and most importantly will mediate reexpression of genes that induce chemosensation. In this study, we define the responsiveness of DLBCL cells to DNMTIs, show that these drugs can indeed enhance the response to chemotherapy, and identify a molecular pathway silenced through aberrant DNA methylation that contributes to this effect in both cell lines and primary human specimens. Furthermore, we show that combination treatment with the DNMTI azacitidine and standard chemomunotherapy is feasible and that DNMTI therapy results in restoration of this silenced pathway and sensitization of lymphoma to chemotherapy in patients.

RESULTS

Decitabine Induces Demethylation and Growth Suppression in a Subset of DLBCL Cells

As a single agent in humans, decitabine (5-aza-2′-deoxycytidine, DAC) is administered at a dosage up to 30 mg/m²/d that is equivalent to approximately 1 μmol/L plasma peak concentration, enough to demethylate cells in susceptible tumors (15). To characterize the responsiveness of a genetically diverse set of DLBCL cells to DNMTI, a panel of 30 DLBCL cell lines was exposed to increasing concentrations of decitabine. The concentration of decitabine that inhibited cell line growth by 50% (GI50) within 48 hours exceeded 2 μmol/L in all the cell lines (Fig. 1A). There were, however, eight cell lines in which the GI50 was lower than 1 μmol/L, ranging from 100 nmol/L in SU-DHL5 and OCI-Ly7 cells to 830 nmol/L in OCI-Ly19 cells (Fig. 1A; Supplementary Table S1); we therefore considered these cell lines to be in vitro sensitive to decitabine. To determine whether differential sensitivity correlated with intracellular drug concentration, five cell lines (DoHH2, SU-DHL6, OCI-Ly1, SU-DHL4, and OCI-Ly3) representing the spectrum of decitabine sensitivity were examined for uptake and cellular retention of [3H]-DAC. We found no association between intracellular [3H]-DAC concentration at 24 or 48 hours with their GI50 for decitabine (Fig. 1B). The expression of membrane transporters and nucleoside transporters that have been suggested to influence the expression of other tumor types (15) was not associated with the response to decitabine (Supplementary Table S1), suggesting that the difference in sensitivity may be biologic in nature.

To determine whether the doses of decitabine required to induce a biologic response in DLBCLs might be linked to the DNA-damaging effects of the DNMTIs, we exposed the more sensitive DLBCL cell lines to their respective GI50 and checked for induction of histone 2AX (H2AX) phosphorylation by flow cytometry. Although decitabine 100 nmol/L, the GI50 for SU-DHL-5 and OCI-Ly7 cells, induced minimal DNA damage (∼5% of cells; Fig. 1C), decitabine 200 and 500 nmol/L, the GI50 for other cell lines, caused marked DNA damage in SU-DHL-5, OCI-Ly7 (Fig. 1C), DoHH2, and SU-DHL-7 (not shown). In OCI-Ly7 cells, decitabine 100 nmol/L at 48 hours was enough to induce biologic changes, as the global 5-methylcytosine content decreased from 15% to 10.2% (Fig. 1D), with a concomitant increase in the activity of caspase-7 and -3 (Fig. 1E). These data indicate that low doses of decitabine can induce DNA demethylation and cell death with minimal DNA damage in susceptible DLBCL cells. We next tested the effect of decitabine in vivo in severe combined immunodeficient (SCID) mice bearing human lymphoma OCI-Ly7 xenografts. Cohorts of mice were treated with vehicle (distilled water and PBS, n = 7) or decitabine 15 mg/m² (n = 5) administered for 5 consecutive days starting on day 1. Treatment was initiated when tumors reached 75 to 100 mm³. Mice were followed until untreated tumors reached 1,000 mm³. This dose of decitabine significantly suppressed the growth of lymphoma xenografts (P < 0.001, t test at day 9; Fig. 1F), causing less than 4% DNA damage (measured by phospho-H2AX immunohistochemistry; Fig. 1G). In contrast, the administration of doxorubicin 0.6 mg/kg caused H2AX phosphorylation in about 20% of the cells (Fig. 1G). Notably, the effect of decitabine was delayed until after the 5-day treatment ended, and peaked at days 9 to 12 (Fig. 1F), suggestive of an epigenetic reprogramming effect due to progressive loss of DNA methylation in DNMTI-sensitive DLBCL cells similar to that observed in vitro and in patients with myeloid malignancies (19–22). Taken together, these data suggest that although demethylating doses of decitabine can induce cell death in a minority of DLBCL cell lines, a bigger proportion of them are sublethally affected.

Decitabine Potentiates the Antilymphoma Effect of Doxorubicin in Chemosensitive Cells

To explore the possibility that decitabine 100 nmol/L would make sublethally affected lymphoma cells more susceptible to chemotherapy, we first exposed the same 30 cell lines to a panel of drugs with known activity in DLBCL, including doxorubicin, dexamethasone, etoposide, mechlorethamine (representing alkylating agents for in vitro use), and methotrexate. Most cell lines exhibited distinct patterns of responsiveness to DNMTIs and chemotherapy drugs (Fig. 2A). This lack of cross-resistance supports the notion of combining agents to achieve more potent antilymphoma activity. Doxorubicin-containing regimens such as R-CHOP are clinically active and routinely used for the treatment of DLBCL (23–26), and...
Figure 1. Antilymphoma activity of decitabine (DAC) A, scatter plot of the GI<sub>50</sub> and GI<sub>25</sub> values of the panel of 30 DLBCL cell lines treated for 48 hours with decitabine. The bottom line represents the dose that causes significant DNA damage and the top line represents the maximum human serum concentration of decitabine. B, <sup>3</sup>H-DAC uptake at 24 and 48 hours in a panel of DLBCL cells with dissimilar decitabine GI<sub>25</sub> values (shown between parentheses, in μmol/L). Cells were cultured with 200 nmol/L of <sup>3</sup>H-DAC for the indicated time. Experiment represents triplicates with SEM. C, percentage of cells positive for phospho-H2AX in OCI-L7 and SU-DHL-5 cells treated for 48 hours with decitabine 100, 200, and 500 nmol/L. D, effect of decitabine 100 nmol/L 48 hours on 5′-methylcytosine content (in %) as determined by high-performance liquid chromatography-mass spectrometry in OCI-Ly7 cells. E, caspase 7 and 3 activity (RLU) determined in OCI-Ly7 cells exposed to decitabine 100 nmol/L for 24 hours or vehicle. F, tumor growth curve of OCI-Ly7 xenografts in SCID mice treated with vehicle (n = 7, water) or decitabine 15 mg/m<sup>2</sup> per day for 5 days (n = 5). P value at day 9 (t test). G, immunohistochemistry (IHC) for phospho-H2AX in the lymphoma tissues of the mice from F and doxorubicin-treated mice for comparison. ×40 photomicrographs (bar = 400 μm, top) and ×100 photomicrographs (bar = 100 μm, bottom). The columns represent the quantification of five slides per mouse segregated by treatment arm and compared with vehicle-treated mice as controls.
Decitabine (DAC) synergizes with doxorubicin (Doxo) in sensitive cells. A, normalized heatmap representation of the GI₂₅ or GI₅₀ values (for decitabine) of the panel of 30 DLBCL cell lines treated for 48 hours by five chemotherapy drugs with effect on DLBCL. Cells are ranked (from lowest to highest, from top to bottom) on the basis of GI₂₅ values for decitabine. Gray scale is normalized for each drug.

B, plot of doxorubicin log GI₅₀ values (y-axis) versus decitabine log GI₉₀ values (x-axis) in a panel of 12 cell lines. The dotted green line represents 250 nmol/L as the cutoff to define sensitivity (bottom portion) versus resistance (top portion) to decitabine in cell lines. The degree of sensitivity to decitabine is represented by gray scale as in A, E, C-dose reduction isobolograms (GI₉₀) for four decitabine- and doxorubicin-sensitive DLBCL cell lines (SU-DHL8, DoHH2, OCI-Ly7, and WSU-NHL) tested for the combination of these drugs. Squares represent each cell line with their relative position to the additive line indicating the result from the combination.

C, graphical representation of the combinatorial effect of decitabine and doxorubicin in sensitive cell lines after exposure to decitabine for 48 hours compared with their respective vehicle-treated cells. Change in GI₉₀ is represented by fold to vehicle. The red arrow indicates the favorable dose reduction zone. Data represent mean of triplicate experiments. E, activity of caspase-7/3 in decitabine- and doxorubicin-sensitive DLBCL cells exposed to vehicle, doxorubicin 100 nmol/L, decitabine 100 nmol/L, or the concomitant combination of drugs for 24 hours. Data is presented relative to vehicle and normalized to the number of cells. F, area under the curve (AUC) of tumor growth curves in OCI-Ly7 xenografted mice treated with vehicle (PBS), decitabine 0.6 mg/kg twice a week (n = 5), decitabine 15 mg/m² daily (n = 5), or their combination (n = 5). Treatment was initiated when tumors reached approximately 100 mm³. Drugs were administered for 10 days, and mice were further followed without treatment until the end of the experiment at day 20. Left, AUC from day 1 till day 10 with all the mice alive. P values represent the comparison of tumor AUC volumes with vehicle. Right, AUC of surviving mice from day 10 to day 20. P values represent the comparison of tumor AUC volumes among treatments. Data are presented as mean with 95% confidence interval. G, graphical representation of the combinational effect of decitabine and doxorubicin in five primary DLBCL cases. CD19⁺ DLBCL cells were exposed to decitabine, doxorubicin, and the combination of drugs in two dose levels. The combinational effect was calculated using the fractional product method and is represented in the y-axis as the mean of four replicates with SEM.
we therefore focused on the pharmacodynamics of combination therapy of DNMTIs with doxorubicin. Individual sensitivity to each drug is a prerequisite to define synergy (27). Sensitivity to doxorubicin was established based on clinically achievable concentrations (∼200 nmol/L) of the dose administered in the CHOP regimen (50 mg/m²; ref. 28). Four decitabine-sensitive cell lines (OCI-Ly7, DoHH2, SU-DHL8, and WSU-NHL) were also sensitive to doxorubicin (Fig. 2B). Exposing these cells to decitabine and doxorubicin resulted in synergistic cell killing in all four cell lines (Fig. 2C). Synergy was also observed for the other classes of drugs included in the CHOP regimen (e.g., vincristine, dexamethasone, and mechlorethamine; Supplementary Fig. S1). Moreover, the addition of decitabine to the four CHOP chemotherapy drugs together allowed for up to a 5.6-fold reduction in the CHOP dose as determined by the dose-reduction index (ref. 27; Fig. 2D).

The effect of decitabine and doxorubicin was independent of whether the drugs were administered sequentially or concurrently (Supplementary Fig. S2A and S2B). Synergistic killing was at least partially due to induction of apoptosis, as coadministration of the two drugs induced greater caspase-7/3 activity (Fig. 2E) and morphologic changes consistent with apoptosis (not shown). Cell-cycle arrest increased with the combination only in WSU–NHL cell lines (Supplementary Fig. S2C), and plasmacytic differentiation markers increased in all the cell lines with the combination (Supplementary Fig. S2D). The efficacy of decitabine and doxorubicin in combination was next tested in vivo in SCID mice bearing human lymphoma OCI–Ly7 xenografts. Cohorts of mice were treated with vehicle (distilled water and PBS, n = 7), doxorubicin 0.6 mg/kg (n = 5), decitabine 15 mg/m² (n = 5), or their combination (n = 5). Treatment was initiated when tumors reached 75 to 100 mm³. Doxorubicin was administered twice a week for 2 weeks (four doses in total) and decitabine was administered for 5 consecutive days starting on day one. Mice were followed for 30 days or until tumors reached 1,000 mm³, and the area under the curve for tumor growth was calculated. At day 10, although doxorubicin suppressed the growth of lymphoma xenografts (P = 0.002, t test; Fig. 2F), after 20 days of follow-up, it was evident that mice treated with the combination showed slower progression of tumor growth compared with decitabine alone (P = 0.025, t test; Fig. 2F).

To determine whether our results obtained in DLBCL cell lines could be extended to primary human DLBCL cells, we obtained single-cell suspensions from the biopsies of five confirmed unselected patients with DLBCL. CD19-positive DLBCL cells were isolated and cocultured with a feeder layer of HK dendritic cells in a dual chamber. Each primary DLBCL specimen was exposed to decitabine 100 or 300 nmol/L, doxorubicin 600 or 1,200 nmol/L, and the combination of the drugs in duplicate (along with four replicates for control and vehicle-treated cells). After 48 hours of exposure, cell proliferation was determined by a metabolic assay. Consistent with the low proliferation rate (5%–20%), and therefore low drug incorporation, there was little response of these specimens to decitabine alone (Supplementary Fig. S3). However, doxorubicin yielded between 10% and 76%, and 7% and 96%, loss of viability after 600 and 1,200 nmol/L concentrations, respectively (Supplementary Fig. S3). To calculate synergy, we used the fractional product method (29). This method is appropriate for the evaluation of a combination of drugs with dissimilar mechanisms of action (such as epigenetic and cytotoxic agents), administered in combination at predefined doses (29). We found that combination treatment was synergistic in four of five cases at 600 nmol/L and five of five cases at 1,200 nmol/L of doxorubicin (Fig. 2G). Therefore, for chemosensitive DLBCL cells, combination therapy with decitabine and doxorubicin resulted in enhanced therapeutic efficacy in vitro and in vivo.

Prolonged Decitabine Administration Induces Reprogramming of Refractory DLBCL Cells

We next examined whether DNMTI could restore responsiveness to doxorubicin in chemotherapy-resistant DLBCL cells. The OCI-Ly1 and Karpas422 cell lines were obtained from patients with DLBCL who failed to respond to several chemotherapy regimens (30, 31) and maintained their resistant phenotype when propagated in vitro (Fig. 2B), therefore representing refractory DLBCLs. The GI₅₀ of decitabine for these chemoresistant DLBCL cells was in the low micromolar range (Fig. 1A) known to be associated with DNA-damaging (off-target) effects (32). Accordingly, exposure of OCI-Ly1 and Karpas422 refractory DLBCL cell lines to GI₅₀ range decitabine caused evident H2AX phosphorylation similar to that induced by doxorubicin (Supplementary Fig. S4A). To ascertain whether decitabine induces chemosensitization independent of DNA damage in refractory DLBCL cell lines, we administered DNMTI at a sub-GI₅₀ dose (i.e., 100 nmol/L) known to cause minimal DNA damage (Fig. 1C) for 5 days, as epigenetic reprogramming induced by DNMTI may take a longer time to manifest (22). This exposure had little impact on H2AX phosphorylation (Fig. 3A), but did induce hypomethylation and decreased the growth rate in both cell lines (Fig. 3B and C), increasing doubling time from 39 to 61 hours and from 50.6 to 67 hours in OCI-Ly1 and Karpas422 cells, respectively. These changes were accompanied by induction of senescence-associated morphologic changes and β-galactosidase activity in OCI-Ly1 cells from 4% at baseline to 68% at day 5 and from 2% to 34% at the same time point in Karpas422 cells (Fig. 3D), with similar increases observed by immunoblotting (Supplementary Fig. S4B). In addition, there was an increase in PARP cleavage, suggesting induction of apoptosis as well (Supplementary Fig. S4B). On the other hand, there was no evidence of differentiation (measured by Cd38+ and Cd138+ flow cytometry; Supplementary Fig. S4C) nor cell-cycle arrest (Supplementary Fig. S4D). These features are consistent with the recently characterized senescence-like phenotype termed senescence with incomplete growth arrest (SWING; ref. 33), in which cells proliferate at a slower rate. Another component of the SWING phenotype is the upregulation of CDKN1A (33), which we also found to be induced at the mRNA (Fig. 3E) and protein (Supplementary Fig. S4E) levels in these cell lines treated with 100 nmol/L of decitabine for 5 days. Development of this particular phenotype is further supported by an increase in p16 (CDKN2A; Supplementary Fig. S4E). SWING is associated with increased sensitivity to doxorubicin-induced genotoxic stress (33), a feature that could be capitalized on for chemosensitization.
**Decitabine Enables Refractory DLBCL Cells to Regain Chemosensitivity**

OCI-Ly1 and Karpas422 cells tolerate doxorubicin-induced DNA damage (H2AX phosphorylation; Fig. 3A) with relatively little cell death (18% and 26% respectively). Accordingly, induction of caspase-7 and -3 activity was observed only at doxorubicin doses of 600 nmol/L or more in OCI-Ly1 cells and 300 nmol/L in Karpas422 cells (Fig. 4A), which exceed the serum concentration commonly achieved in the CHOP regimen (i.e., ~200 nmol/L; ref. 28). In contrast, pretreatment of these cells with the five-day 100 nmol/L dosing schedule of decitabine induced caspase-7 and -3 cleavage even with a concentration as low as 37.5 nmol/L of doxorubicin (Fig. 4A) and decreased the doxorubicin GI50 by 5.3- and 15-fold in OCI-Ly1 and Karpas422 cells, respectively (Fig. 4B). Chemosensitization was likewise observed with azacitidine (not shown). A similar effect was observed for the two other doxorubicin-resistant cell lines SC1 and RL (Fig. 4B). To determine whether DNMTIs enhance the effect of doxorubicin in a resistant DLBCL cell line in vivo, we treated OCI-Ly1 cell xenografted mice (n = 31) with a sequential schedule. Once tumors reached 75 to 100 mm³, mice were randomized to three groups and treated with vehicle (n = 8, PBS), doxorubicin 0.6 mg/kg (n = 8), or decitabine 15 mg/m² (n = 15). After 10 days, the 15 decitabine-treated mice were again randomized to vehicle (n = 8, PBS) or doxorubicin (n = 7). During the 20-day period, animals in the doxorubicin-only arm were administered the drug twice a week for a total of five doses, decitabine alone was administered daily for 10 days, and the combination consisted of 10 consecutive days of decitabine and one dose of doxorubicin. In this case, tumor growth was indeed more potently suppressed by the decitabine–doxorubicin combination compared with each drug alone (Supplementary Fig. S5A), suggesting that epigenetic reprogramming drugs may also require time to sensitize tumors to DNA-damaging agents in vivo. A second cohort of OCI-Ly1 xenografted SCID mice (n = 31) was therefore treated with a sequential schedule. Compared with the CHOP regimen (i.e., ~200 nmol/L; ref. 28), decitabine alone was administered daily for 10 days, and the combination consisted of 10 consecutive days of decitabine and one dose of doxorubicin. In this case, tumor growth was indeed more potently suppressed by the decitabine–doxorubicin combination compared with each drug alone (P = 0.008 for decitabine and P < 0.001 for doxorubicin; Fig. 4C). This regimen caused no increase in DNA damage (measured by phosphorylation of H2AX) in tumors treated with decitabine as compared with vehicle alone (Fig. 4D), but increased the DNA damage caused by doxorubicin alone (Fig. 4D). There was no evidence of toxicity to normal tissues based on histologic examination (not shown).
Figure 4. Five-day exposure to decitabine (DAC) sensitizes cells to doxorubicin (Doxo). A, activity of caspase-7/3 (y-axis, fold to control) in OCI-Ly1 and Karpas422 cells preexposed to vehicle or decitabine 100 nmol/L for 5 days and treated with water (UT) or five concentrations of doxorubicin. B, dose reduction plot for doxorubicin GI50 (fold decitabine to vehicle, x-axis) in the panel of eight doxorubicin-resistant cell lines after exposure to decitabine for 5 days compared with their respective vehicle-treated cells. Change in GI50 is represented by fold to vehicle. The red arrow indicates the favorable dose reduction zone. Data represent mean of triplicate experiments. C, AUC of tumor growth curves in OCI-Ly1 xenografted mice treated with vehicle (V; PBS, n = 8), doxorubicin 0.6 mg/kg twice a week (n = 8), decitabine 15 mg/m2 daily (n = 8), or their sequential combination (n = 7). Treatment was initiated when tumors reached approximately 100 mm3, drugs were administered sequentially during the first 10 days, and mice were further followed without treatment until the end of the experiment at day 20. P values represent the comparison of tumor AUC volumes with vehicle and between decitabine and the combination. D, immunohistochemistry (IHC) for phospho-H2AX in the lymphoma tissues of the mice from C. x40 photographs (bar = 400 μm; left) and x100 photographs (bar = 100 μm; right). Quantification of positive cells (in %) is shown as inserts. E, CD19+ single-cell suspensions from lymph node biopsies of four refractory DLBCL specimens were exposed to decitabine 100 nmol/L for 48 hours followed by either doxorubicin 0.6 μmol/L, doxorubicin 1.2 μmol/L, or vehicle for additional 48 hours. Cell viability at 96 hours (normalized to 48 hours of vehicle or decitabine-treated cells) is shown on the y-axis. The experiment was carried out in duplicate.
suggesting that the combination can be safely administered. To further explore toxicity, we administered the same regimen to immunocompetent C57BL/6 mice (n = 5 combination and 5 vehicle control). We again could not detect any evidence of organ damage by tissue macroscopic examination and microscopic analysis including bone marrow, liver, and heart (Supplementary Fig. SSB). No animals died. One mouse had a decrease in total white blood cell counts without evidence of bone marrow damage, and all mice presented elevation of total bilirubin levels without evidence of liver toxicity by microscopic examination (Supplementary Fig. SSB and SSC).

We next obtained single-cell suspensions from the biopsies of four clinically documented relapsed and chemoresistant DLBCL cases. CD19-isolated cells were sequentially exposed to decitabine 100 nmol/L or vehicle for 48 hours, followed by either doxorubicin 600 nmol/L, doxorubicin 1,200 nmol/L, or vehicle for an additional 48 hours. Notably, all cases were resistant to doxorubicin 600 nmol/L, and two cases were also resistant to doxorubicin 1,200 nmol/L (Fig. 4E). However, after decitabine 100 nmol/L, all the samples were more sensitive to doxorubicin 600 nmol/L and three samples were more sensitive to doxorubicin 1,200 nmol/L (Fig. 4E). Therefore, DNMTIs can safely overcome chemotherapy resistance in vitro and in vivo in cell lines and primary DLBCLs and may represent a potential therapeutic strategy to improve outcome for patients with high-risk disease.

**SMAD1 Methylation Associates with Chemoresistant Phenotypes in DLBCL**

To explore the nature of the epigenetically encoded chemoresistance in DLBCL cells, we first measured intracellular accumulation of anthracycline in our eight doxorubicin-resistant cell lines. All the chemoresistant cell lines accumulated intracellular anthracycline to levels similar to doxorubicin-sensitive cells (Supplementary Fig. S6), which was approximately one order of magnitude higher than the cells engineered to express a multidrug resistant (MDR) phenotype (Supplementary Fig. S6). We hence postulated that chemoresistance might be epigenetically mediated. To identify candidate epigenetically silenced genes that might contribute to doxorubicin resistance, we conducted gene expression and DNA methylation profiling using the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) assay of a panel of eight doxorubicin-resistant versus six doxorubicin-sensitive cells (from Fig. 2A; Supplementary Table S1). Nine unique genes were found to be both reproducibly and specifically hypermethylated and repressed in resistant cell lines (fold difference > 1.5, representing ~20% difference in methylation; ref. 12; Fig. 5A; Supplementary Table S2). To determine whether these genes were in fact silenced by DNA methylation, the chemoresistant cell lines OCI-Ly1 and Karpas422 were treated with decitabine 100 nmol/L for 5 days and transcript abundance measured by quantitative PCR (qPCR). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HPRT (hypoxanthine phosphoribosyltransferase) were used as controls (non-differentially methylated or expressed). Among nine genes, we found that VAV3, ETV6, and SMAD1 were the most upregulated with more than fivefold induction (Fig. 5B). SMAD family proteins mediate the actions of TGFβ in suppressing lymphocyte proliferation and inducing differentiation and apoptosis (34). SMAD1/5/8 also mediates chemotherapy-induced senescence in cancer cells (35, 36), a phenotype that we observed in OCI-Ly1 and Karpas422 cells treated with a low dose of decitabine (Fig. 3D and Supplementary Fig. S4). Quantitative DNA methylation analysis (Sequenom Epityping) of the SMAD1 locus confirmed the increased CpG methylation status of this locus in doxorubicin-resistant cell lines OCI-Ly1, Karpas422, and OCI-Ly10 versus chemosensitive OCI-Ly7 cells (Fig. 5C). To determine whether this association was also present in patients, we analyzed SMAD1 methylation status in 22 primary DLBCL cases, of which 11 were refractory and 11 sensitive to R-CHOP. We found an increase in CpG methylation of SMAD1 in the refractory versus sensitive cases (Fig. 5D; P = 0.016, Mann–Whitney test). To determine the prevalence of SMAD1 methylation in patients with DLBCL, we conducted HELP assays in a cohort of 231 unselected cases. We found that 75% of patients with DLBCL showed 5% or less SMAD1 methylation (Fig. 5E). Reciprocally, 70% of the cases from a cohort of 248 patients with DLBCL showed phospho-SMAD1 expression in 20% or more cells by immunohistochemistry using tissue microarrays (Supplementary Fig. S7). These proportions are overall consistent with the frequency of patients who initially respond to chemotherapy (17). To further determine the association of SMAD1 with chemoresistance phenotype, we analyzed SMAD1 methylation and expression according to DLBCL cell of origin status (i.e., GCB vs. ABC type, the latter of which are more chemotherapy resistant). The databases included (i) a publicly available DNA methylation profiling dataset consisting of 57 newly diagnosed DLBCLs (40 GCB and 17 ABC) treated with R-CHOP (13) and (ii) two publicly available DLBCL gene expression microarray datasets including 201 DLBCLs (108 GCB and 93 ABC; ref. 37) and 119 DLBCLs (85 GCB and 34 ABC; ref. 38), respectively. We observed that SMAD1 was more frequently highly methylated in ABC versus GCB DLBCLs (P = 0.018, t test; Fig. 5F) and was also expressed at lower levels in ABC versus GCB cases (7.3 ± 0.8 vs. 8.4 ± 1, respectively; P = 2.24 × 10⁻³ and 5.1 ± 0.14 vs. 5.8 ± 0.14, respectively; P = 0.001, t tests; Fig. 5G). These data suggest that in patients with DLBCL, SMAD1 hypermethylation and lower expression are associated with a more chemoresistant phenotype.

**Reactivation of SMAD1 Contributes to Chemosensitization of DLBCL Cells**

We next wished to determine whether SMAD1 reactivation contributes to chemosensitization induced by decitabine. Exposure of OCI-Ly1 and Karpas422 cells to decitabine 100 nmol/L for up to 120 hours induced 5% to 18% reduction of SMAD1 methylation (Fig. 6A) for most CpGs, concordant with the increase in SMAD1 transcript abundance. A similar SMAD1 demethylation effect was detected in SC-1 cells (Fig. 6A). The area more susceptible to demethylation included an upstream CpG shore (39, 40) of 500 bp and a CpG island encompassing the SMAD1 promoter region to exon 2 (Fig. 6A). To determine the stability of the effect of decitabine on SMAD1 demethylation, DLBCL cells were treated as before (decitabine 100 nmol/L for 5 days), followed by a decitabine washout period of 5 days, and analyzed for SMAD1 methylation. During the washout period, cells continued to proliferate at a much lower rate than untreated cells, increasing doubling time from 39 (untreated) to 58 hours and from 50.6 (untreated)
Figure 5. SMAD1 is methylated in DLBCLs with chemoresistant phenotype. A, intersection plot showing nine common hypermethylated (HELP > 1.5) and low abundance (GE < 1.5) genes from a panel of doxorubicin-resistant DLBCL cell lines. B, transcript abundance (fold to vehicle, y-axis) of nine hypermethylated and low abundant genes in Karpas422 and OCI-Ly1 cells treated with 100 nmol/L of decitabine for 72 and 120 hours. HPRT and GAPDH are negative controls. C, representation of SMAD1 gene methylation in DLBCL cell lines with distinct sensitivity to doxorubicin (more sensitive on top, more resistant on bottom). Data are presented as percent of methylation (0%–100%) of a gene region accordingly to the RefSeq representation. D, representation of SMAD1 gene methylation in DLBCL patient specimens with distinct sensitivity to R-CHOP (11 responsives vs. 11 refractories). Data are presented as the percentage of methylation (median with interquartile range) for each group. E, SMAD1 methylation determined by HELP assay in 231 patients with DLBCL. Bars represent the frequency of patients per SMAD1 methylation category. Number of patients per category is shown on top. F, graphical representation of SMAD1 methylation (HpaII/MspI ratio, y-axis) of 17 ABC DLBCL cases versus 40 GCB DLBCL cases. The red line represents the mean. P values for the comparison between ABC and GCB cases are shown on top. G, gene expression probeset intensity for SMAD1 between 93 ABC DLBCL cases and 108 GCB DLBCL cases (left) and between 34 ABC DLBCL cases and 85 GCB DLBCL cases (right). The red line represents the mean. P values for the comparison between ABC and GCB cases are shown on top.
Figure 6. Reactivation of epigenetically silenced SMAD1 contributes to chemosensitization. A, representation of the change in the methylation (in %) of SMAD1 in chemoresistant OCI-Ly1, Karpas422, and SC-1 cells after treatment with decitabine for 72 hours, 120 hours, and 120 hours followed by a washout period of 120 hours (w.o.) compared with baseline methylation levels. B, immunoblot for SMAD1 (and GAPDH as control) for cells with dissimilar GI50 for doxorubicin (top). The ratio for the density of SMAD1 over GAPDH is shown at the bottom. C, immunoblot for SMAD1 in OCI-Ly1 and Karpas422 cells treated with vehicle (0 h) or decitabine for 72 and 120 hours. Whole-cell lysate and nuclear fraction are shown on top and bottom, respectively. GAPDH for whole-cell lysates and histone 3 for nuclear extracts were used as controls. D, cell viability of OCI-Ly1 and Karpas422 cells treated with vehicle or decitabine 100 nmol/L for 72 and 120 hours and treated with TGFβ1 and BMP2 for additional 24 hours. Data represent experimental triplicates. E, Differential viability of OCI-Ly1 cells transfected with SMAD1 or empty-vector (EV) plasmids and treated with water (vehicle), TGFβ1, or doxorubicin 600 nmol/L for 24 hours. Experiment represents the mean of triplicates with SEM. F, cell viability determined in OCI-Ly1, Karpas422, SC-1, and OCI-Ly10 cells transfected with SMAD1 2 μg or empty vector 2 μg (EV) for 24 hours and subsequently treated with doxorubicin 600 nmol/L or vehicle for additional 24 hours. Results are relative to EV + doxorubicin 600 nmol/L set as 100% viability. G, viability of OCI-Ly1 cells after 48 hours of transfection with si-NT or si-SMAD1 #7 and treated with vehicle (UT) or several concentrations doxorubicin for 24 hours (10, 30, 125, 250, and 500 nmol/L).
Epigenetic Chemosensitization of Aggressive Lymphomas

We used a modified Continuous Reassessment Model to determine dose escalation. Enrollment has been completed with 12 patients treated (Fig. 7A). Eleven of 12 patients have achieved a complete response (Fig. 7A and B) and 10 remain in remission at median follow-up of 13 months (range 5 to 28 months). One of the patients who relapsed had discontinued both azacitidine and rituximab after two treatment cycles due to hepatitis C reactivation.

We observed an association between SMAD1 protein abundance and doxorubicin sensitivity (Fig. 6B) and a concurrent increase in nuclear SMAD1 protein abundance in Karpass422 and OCI-Ly1 cells exposed to 100 nmol/L decitabine (Fig. 6C).

In DLBCL and FL cells, SMAD1, which is normally associated with bone morphogenetic protein (BMP) signaling, is aberrantly activated following TGFβ stimulation (41). Loss of TGFβ antiproliferative response, normally occurring in T and B lymphocytes (42, 43), has been reported in NHLs, specifically in T-cell lymphomas (44, 45) and small lymphocytic lymphoma/chronic lymphocytic leukemias (42). OCI-Ly1 and Karpass422 cells grown in serum-depleted medium showed a modest response to the inhibitory effect of BMP2 and TGFβ1. However, exposure to 100 nmol/L of decitabine for up to 5 days sensitized these cells, in a time-dependent manner, to the inhibitory effect of these ligands by approximately 20% to 35% in Karpass422 cells and approximately 20% to 60% in OCI-Ly1 cells compared with vehicle (Fig. 6D and Supplementary Fig. S8A). In addition, OCI-Ly1 cells engineered to overexpress SMAD1 to levels similar to those induced by DNMTI displayed reduced proliferation in a dose-dependent manner (Supplementary Fig. S8B). After 4 days of transfection with SMAD1, OCI-Ly1 cells decreased their proliferation rate by 2.6 times (Supplementary Fig. S8C). Moreover, overexpression of exogenous SMAD1 in these refractory cells (OCI-Ly1) rendered them more susceptible to the inhibitory effects of TGFβ1 and doxorubicin (Fig. 6E). A similar effect was found in Karpass422 and SC-1 cells and with lower magnitude in OCI-Ly10 cells (Fig. 6F). Conversely, SMAD1 knockdown further increased the resistance to doxorubicin in OCI-Ly1 cells (Fig. 6G and S9A) and, to a lesser extent, in Karpass422 cells (Supplementary Fig. S9B). Taken together, these results suggest that epigenetic silencing of SMAD1 confers a growth advantage and contributes to chemoresistance of DLBCL cells.

Combination of DNMTI with R-CHOP Is Feasible in Newly Diagnosed High-Risk DLBCL Patients

On the basis of our preclinical results, we initiated a phase 1 clinical trial in patients with newly diagnosed DLBCL to test the feasibility, efficacy, and biologic effects of 5-day exposure to DNMTI (in this case azacitidine) administered before R-CHOP. Azacitidine was administered subcutaneously at escalating doses (25 mg/m², 50 mg/m², or 75 mg/m² daily for 5 days), followed by standard R-CHOP chemotherapy administered on day 8, with cycles repeated every 21 days for six cycles. We used a modified Continuous Reassessment Model to determine dose escalation. Enrollment has been completed with 12 patients treated (Fig. 7A). Eleven of 12 patients were of high-intermediate risk or high risk by the IPI, and one patient was of low-intermediate risk. Median age was 65 years (range 43–83 years). All but one patient were over the age of 60 years. The combination therapy was well tolerated. Two of 10 patients treated at the highest dose level experienced dose-limiting toxicity (DLT): one developed reactivation of hepatitis C and the other experienced prolonged neutropenia, which subsequently resolved. All patients experienced transient grade 3 or 4 neutropenia, and four patients developed grade 3 neutropenic fever. Six patients underwent modification of planned therapy. In addition to the two patients who developed DLT, who discontinued azacitidine at the time of these events, one patient refused the last cycle of R-CHOP, another refused the last cycle of azacitidine, one patient discontinued azacitidine (but completed planned R-CHOP) after experiencing a gastrointestinal hemorrhage related to lymphoma response in a large gastric mass, and one patient had a 3-day dose delay due to grade 2 thrombocytopenia on the first day of cycle 3 (Fig. 7A). Eleven of 12 patients have achieved a complete response (Fig. 7A and B) and 10 remain in remission at median follow-up of 13 months (range 5 to 28 months). One of the patients who relapsed had discontinued both azacitidine and rituximab after two treatment cycles due to hepatitis C reactivation.

We obtained CD19-purified DLBCL cells from the serial biopsy specimens of patients who consented to this procedure immediately before the first treatment and after the fifth day of treatment with azacitidine. Patient 1 yielded sufficient cells to conduct global cytosine methylation analysis by liquid chromatography/random mass spectrometry at both time points, and exhibited a decrease in global cytosine methylation of a magnitude comparable with that shown for cell lines treated with decitabine in vitro (Fig. 7C, compared with Fig. 1D). In samples from six patients, we were able to measure SMADI methylation by Sequenom Epityping. Compared with the respective pretreatment time points, we observed a decrease in SMADI methylation after treatment with azacitidine in all patients (Fig. 7D). For some patients, enough material was available to determine SMADI expression either by qPCR or immunohistochemistry. These studies yielded a post-azacitidine increase in SMADI mRNA abundance (Fig. 7E) as well as in phospho SMADI protein expression (Fig. 7F). Moreover, the greater yield of cells obtained from patient 1 allowed us to determine whether the administration of azacitidine rendered the tumor more sensitive to chemotherapy. Purified DLBCL tumor cells obtained from the patient pre- and post-azacitidine treatment were exposed ex vivo to doxorubicin, rituximab, and CHOP-like combination chemotherapy, and cell viability was determined after 48 hours of exposure. We observed a large increase in the fraction of dead cells in the post-azacitidine sample compared with the pre-azacitidine sample (Fig. 7G). The chemosensitization effect was particularly evident for doxorubicin, where the excess of killing in the posttreatment sample was 55% for 100 nmol/L of doxorubicin (P < 0.01, paired t test; Fig. 7G). Taken together, our results show that sequential DNMTI followed by R-CHOP can be safely administered to newly diagnosed older patients with high-risk disease, resulting in high rates of complete clinical response, and confirm the chemosensitization effect identified in preclinical studies associated with reactivation of SMADI.

DISCUSSION

One major barrier to the eradication of DLBCL with anthracycline-based chemotherapy is the ability of certain lymphomas to exhibit resistance to these drugs. Anthracycline
resistance may be intrinsic or acquired and may occur through multiple mechanisms. These mechanisms may be classified in two major groups: (i) decreased drug accumulation and/or increased drug inactivation, for example, via upregulation of the MDR/TAP (multidrug resistance/antigen peptide transporter) genes; this mechanism has been characterized in cell and animal models but its relevance to clinical resistance remains unclear in most tumor types (46–49) and (ii) biologic mechanisms that prevent cell death from occurring after anthracycline-induced damage. Accumulating evidence indicates that the capacity of most cancers to resist the cytotoxic effects of chemotherapy is more closely connected to genetic and epigenetic abnormalities that affect critical cell-cycle checkpoint and cell death pathways than to specific mechanisms of resistance unique to each agent (50, 51). Restoring nonfunctional pathways in certain tumor cells may increase their susceptibility to cancer chemotherapy more efficiently than improving drug–target interactions. For example, abrogation of the function of the tumor suppressor p53 may compromise the capacity of lymphoma cells to induce cell-cycle arrest and/or to trigger apoptosis in response to DNA-damaging agents (6, 52, 53). Along these lines, herein we show that the TGFβ pathway transducer SMAD1 is silenced through aberrant DNA methylation, and that this silencing contributes to chemotherapy resistance in DLBCL.

TGFβ belongs to a superfamily of polypeptide growth factors that control the growth, proliferation, differentiation, and apoptosis of normal and malignant cells of multiple lineages (34). Normal and neoplastic B cells have receptors for TGFβ and can synthesize and secrete several of these ligands (34, 54). The cellular actions of the various TGFβ receptors are mediated through SMAD proteins, among which SMAD1 and SMAD5 are preferentially involved in the BMP-dependent...
pathways, whereas SMAD2 and SMAD3 are involved in the TGFβ pathway (54). However, noncanonical activation can lead to the formation of mixed complexes (34, 54, 55). With the exception of certain Epstein-Barr virus–positive B-cell lymphomas, the loss of the TGFβ (and BMP) antiproliferative response is a hallmark in NHLs (41, 54). Although genetic mutations in SMADs are rare in hematologic malignancies, a recent publication suggested that SMAD5 could be silenced through miR-155 in DLBCLs (41). Our results indicate that in DLBCL, the other member of the complex, SMAD1, could be epigenetically silenced through aberrant DNA methylation. BMP6, another component of the TGFβ SMAD1/5/8 pathway, was also found to be hypomethylated in patients with DLBCL (8). Other transcriptional mechanisms may impair TGFβ signaling in DLBCL; for example, the transcriptional repressor BCL6 was found to disrupt the SMAD4/p300 interaction and repress SMAD4 in Burkitt Lymphoma (56). DNA methylation-mediated repression of SMAD1 is functionally relevant to chemoresistance, as treatment of DLBCL cells with low-dose long-term decitabine derepressed SMAD1 and restored responsiveness to the growth-inhibitory effect of TGFβ signaling and chemotherapy agents. Moreover, a similar effect was induced by ectopically expressing SMAD1 in these cells. Conversely, downregulation of SMAD1 was sufficient to partially rescue DLBCL cells from decitabine-induced anti-lymphoma effects.

We noted that low-dose decitabine induced a senescence-like phenotype in DLBCL cells. Stress-induced and replicative senescence constitute strong antiproliferative responses to protect normal cells from oncogenic events (57). Senescent cells are physiologically characterized by terminal growth arrest and exhibit particular morphologic and biochemical features (57). Some of these features, such as expression of senescence-associated β galactosidase, are also induced upon chemotherapy treatment of cancer cells. The role of senescence in the response to chemotherapy treatment is controversial, as these cells are terminally arrested (not proliferating), making them apparently less susceptible to the effect of classical chemotherapy drugs that target proliferating cells (57). However, several preclinical studies showed that tumor cells with a senescence phenotype are more susceptible to genotoxic agents (57–60). This may be due to changes in death threshold associated with defective DNA damage response and possibly other effects in senescent cells (57). However, it is also possible that cancer cells activate a different senescence-like program than normal cells. Along these lines, recent studies suggest that activation of oncogenes in tumor cells, which like DLBCL cells express endogenous telomerase activity (61) and commonly lack critical regulators of cell-cycle progression, may be sufficient to trigger morphologic and biochemical manifestations of senescence but cannot induce full growth arrest (33). When this senescence-like program is activated, cells have an impaired DNA damage response, making them more susceptible to genotoxic stresses induced by doxorubicin and other compounds (33). We find that this program, named SWING (33), is induced in DLBCL cells by low doses of decitabine and that these cells exhibited more pronounced DNA damage and cell death when subsequently exposed to chemotherapy treatments. The suppression of the DNA damage response in the SWING phenotype is critically dependent on the CDKNIA pathway (33). In this regard, it is possible that upregulation of CDKNIA upon induction of the SWING phenotype in DLBCL cells depends on the function of SMAD1, as CDKNIA is a target gene of the BMP/SMAD pathway (62).

Despite their efficacy in myelodysplasia (63), the need for optimal dose and schedule as well as pharmacodynamic markers has been a major challenge for clinical translation of DNMTIs for patients with lymphoma following a classical approach for anticancer agents, i.e., the use of maximal tolerated dose, showed a low therapeutic index for these drugs (64). We pursued an alternative approach in our clinical trial: that of using DNMTI therapy to “prime” DLBCL before administration of chemotherapy. We hypothesized that pretreatment with the DNMTI would result in demethylation and reexpression of genes that play a role in modulating chemotherapy sensitivity in the lymphoma cells. We found that the combination was tolerable, even in older patients, with manageable toxicity. Furthermore, preliminary efficacy results were promising in this high-risk population.

Our results suggest a more rational way to incorporate these drugs for clinical use based on their pharmacodynamics in DLBCL cells. Specifically we note that (i) in agreement with a recent publication (22), doses of DNMTIs that induce DNA demethylation are about 10 orders of magnitude lower than those causing significant DNA damage; (ii) gene demethylation followed by phenotypic changes (reprogramming) is critical to chemosensitization; and (iii) marked ex vivo chemosensitization is accompanied by demethylation and expression of specific genes but modest global hypomethylation, suggesting that reactivation of specific pathways (i.e., TGFβ) may be more relevant for the final outcome.

DNA replication and proliferation can also influence the final effect of DNMTIs. The high proliferative rate of DLBCLs offers the opportunity for DNMTIs to readily incorporate into the DNA of these tumor cells and block DNMT activity. Most DLBCL cell lines have doubling times of between 18 and 36 hours; hence, DNMTIs are able to induce chemosensitization in vitro and in vivo. DLBCLs in humans are also highly proliferative, and we accordingly observed a similar chemosensitization effect when the drugs were administered to patients. Ex vivo, primary human DLBCLs proliferate much less, and so the effect of decitabine was less pronounced. As clinical trials of low-dose DNMTIs proceed, it will be important for correlative studies to carefully examine the association between tumor proliferative rates and the level of chemosensitization induced by these drugs. Equally important to the rational deployment of DNMTIs to the clinic is the identification of predictive markers that can guide the selection of these drugs for patients most likely to respond. In this regard, we found SMAD1 hypermethylation in patients with lymphoma to be associated with more chemoresistant subtypes of DLBCLs (ABC-DLBCLs) and poorer overall survival. Whether SMAD1 methylation status will help to predict response to DNMTIs, R-CHOP, or other treatments will require adequately powered prospective clinical trials. The research presented herein provides a mechanism-based rationale for the study of DNMTI in DLBCLs, a biologic explanation (SMAD1 hypermethylation...
and silencing) for chemotherapy resistance and poor outcome in DLBCL, and early-phase clinical therapeutic findings that will guide the design of larger-scale clinical trials exploring this strategy to overcome chemotherapy resistance and potentially improve outcomes for patients with this disease.

**METHODS**

**Cell Lines**

DLBCL cell lines OCI-Ly1, OCI-Ly4, OCI-Ly10, and OCI-Ly7 were grown in medium containing 90% Iscove’s and 10% fetal calf serum (FCS; 20% FCS for OCI-Ly10) and supplemented with penicillin G and streptomycin. DLBCL cell lines DB, DOHH2, Farage, HT, Karpas422, Karpas231, NU-DUL-1, OCI-Ly3, OCI-Ly8, OCI-Ly18, OCI-Ly19, OZ, RL, RC-A8, RI-1, SU-DHL4, SU-DHL5, SU-DHL6, SU-DHL7, SU-DHL8, SU-DHL10, SC1, Toledo, VAL1, WSU-NHL, and WSU-DLBCL-2 were grown in medium containing 90% RPMI and 10% FCS supplemented with penicillin G and streptomycin, l-glutamine, and HEPES. Cell lines were obtained from the American Type Culture Collection, DMSZ, or the Ontario Cancer Institute. We conducted monthly testing for mycoplasma sp. and other contaminants and quarterly cell identification by single-nucleotide polymorphism.

**Single Locus DNA Methylation Assays**

Total genomic DNA was extracted from 4 × 10⁹ DLBCL cells and CD19⁺ patient samples using the PureLink Mini Kit (Invitrogen) and eluted in RNAse-free water. EpiTYPER assays (Sequenom) were conducted on bisulfite-converted DNA. Bisulfite conversion was conducted using the EZ DNA Methylation kit from Zymo Research. EpiTYPER primers were designed to cover CpG islands associated with the respective HpaII amplifiable fragments. All primers were designed using Sequenom Epidesigner beta software (http://www.epidesigner.com/). Primer sequences are shown in Supplementary Table S3.

**Cellular Uptake and Retention of [³H]-DAC**

Cell lines OCI-Ly3, OCI-Ly1, DOHH2, SU-DHL4, and SU-DHL6 were incubated with [³H]-DAC 200 nmol/L in complete medium for 24 and 48 hours when cellular radioactivity was determined in counts per minute. Data are presented normalized to OCI-Ly1 with SEM.

**Cellular Uptake and Retention of Daunorubicin**

Cellular uptake of doxorubicin (hydroxy-daunorubicin) was determined by uptake of daunorubicin as previously published (65). Briefly, cell lines OCI-Ly1, OCI-Ly10, OCI-Ly18, Karpas231, Karpas422, SC1, RL, VAL1, CCRF-CEM/VBL100 (MDR⁺; ref. 66), and CCRF-CEM (MDR⁻) were incubated with vehicle, daunorubicin 500 nmol/L or 2.5 μmol/L in complete medium at 37°C for 1 hour and washed three times with PBS. Fluorescence (PE-A channel) was determined with a flow cytometer (LSAR II BD Biosciences).

**Growth Inhibition Determination**

DLBCL cell lines were grown at concentrations sufficient to keep untreated cells in exponential growth over the complete drug exposure time. Cell viability was determined using a fluorometric resazurin reduction method (CellTiter-Blue, Promega) and Trypan blue automatic method (TC10, Bio-Rad). Fluorescence (Ex485/Fm530) was determined with the Synergy4 microplate reader (BioTek). The number of viable cells was calculated using the linear least-squares regression of the standard curve. The fluorescence was determined for three replicates per treatment condition and normalized to their respective controls (vehicle-treated cells). To plot dose-effect curves, CompuSyn software (Biosoft) was used and drug concentrations that inhibit the growth of the cell lines by 25% and 50% compared with control (GI₂₅ and GI₅₀, respectively) were determined. Data were presented as the mean GI₂₅ or GI₅₀, with a 95% confidence interval for duplicate experiments. Data are normalized to cellular doubling time (Supplementary Table S1). To determine synergism in primary cells, we used the Webb fractional product method (29). This method is based on the equation $Z = X + Y(1 - X)$, where $Z$ is the expected effect of the combination and $X$ and $Y$ represent the effect of each drug alone. If $Z$ is equal to the actual effect of the combination, then the relation is additive; if $Z$ is higher then it is less than additive, and if $Z$ is lower then it is more than additive (synergistic). Data are presented as the mean of four replicates with SEM.

**Array-Based Methylation Analysis**

The HELP assay was conducted as previously published (12). Briefly, one microgram of high molecular weight DNA was digested overnight with isoschizomer enzymes HpaII and MspI (New England Biolabs). DNA fragments were purified with phenol/chloroform, resuspended in 10 mmol/L Tris-HCl pH 8.0, and used immediately to set up the ligation reaction with MspI/HpaII-compatible adapters and T4 DNA ligase. Ligation-mediated PCR was conducted with enrichment for the 200 to 2,000 base pair products and was submitted for hybridization (Roche). We used the HG 17 human promoter custom array covering 25626 HpaII amplifiable fragments within the promoters of the genes. Data quality control and analysis were conducted as described previously. Probe sets with intensity of less than 2.5 mean absolute deviation of the random probe sets on the array were marked as missing values. After quality control processing, a median normalization was conducted on each array by subtracting the median log-ratio (HpaII/MspI) of that array (resulting in median log-ratio of 0 for each array). The HELP array probesets for SMAD1 are M3PI046S00245618 and M3PI046S00245619. Correlation of HELP data with percent of methylation was done as published (12).

**DNA Damage Analysis**

Cells: DNA damage (double-strand breaks) was determined by quantification of phosphorylation of H2AX (Ser139) using a fluorescence-based assay (Millipore) following the manufacturer’s instructions with modification for suspension cells. The nuclear dye (Hoechst 33342) was used to determine cell number for normalization. Data are presented as fold H2AX phosphorylation in treated cells versus vehicle (water) at the indicated exposure times. Tissues: phosphorylation of H2AX (Ser139) (Millipore, clone EP854Y) was determined by immunohistochemistry as previously described (67) with modifications. Briefly, antigen retrieval was conducted in EDTA buffer pH 9 at 95°C (in a steamer) for 15 minutes. Cases were blind scored by two researchers.

**Caspase-7 and -3 Activity**

The activity of caspase-7 and -3 was determined using the ApoONE caspase 3/7 assay (Promega) following the manufacturer’s instructions. DLBCL cells were treated in triplicate with vehicle, TGFβ1, BMP2, doxorubicin, decitabine, or their combinations at indicated concentrations and time points. The presence of caspase-3 and -7 activity allows the R110 group to become intensely fluorescent (Ex485/Em490) which is measured using the Synergy4 microplate reader (BioTek). Caspase-3 and -7 activity was related to the cell number determined by CellTiter-Blue (Promega) in a multiplex assay.

**Cellular Senescence**

Cellular features of senescence were determined in DLBCL cells after 72 and 120 hours of administration of decitabine 100 nmol/L (once per day) or water using the senescence β-galactosidase kit (Cell Signaling) following the manufacturer’s instructions with modifications for suspension cells. Cells were stained at 37°C for at least 6 hours. Pictures were taken using an Olympus DP72 camera in a Zeiss Axioscope microscope. Experiments were carried out in triplicate.
Immunoblotting

Lysates from DLBCL cells were prepared using 50 mM/L Tris pH 7.4, 150 mM/L NaCl, and 1% NP-40 lysis buffer. Lysates for nuclear and cytoplasmatic fractions were obtained using a fractionation kit (Biovision) following the manufacturer’s instructions. Protein concentrations were determined using the BCA Kit (Pierce). Twenty-five to 50 micrograms of protein lysates were loaded in SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and probed with primary antibodies: mouse monoclonal to SMAD1 (Santa Cruz), mouse rabbit polyclonal to GAPDH (Santa Cruz), mouse monoclonal to PARP-1 (F-2; Santa Cruz), mouse monoclonal to p21 (DCS-60; Santa Cruz), and mouse monoclonal to p16 (F-12; Santa Cruz). Densitometry values were obtained by using the Image 1.40g software (NIH, Bethesda, MD).

SMAD1 siRNA

Predesigned siRNA targeting SMAD1 (Invitrogen catalog s8394, s8395, and s8396) and control (a sequence with no significant similarity to any vertebrate gene, Invitrogen 12935-110 Lo GC Duplex 2) were obtained from Invitrogen. Cells were transfected with 2 µmol/L of siRNA by electroporation (Amazza electroporator) using the transfection buffer recommended by the manufacturer (Amazza) or subjected to the procedure without siRNA (mock control).

Transient Transfection of SMAD1 into DLBCL Cells

eGFP (backbone control) or eGFP-SMAD1 plasmids were transfected at several concentrations into DLBCL cell lines by electroporation (Amazza nucleofector, Amazza). Cells were left to recover for 12 hours, reseeded in complete growth medium, and treated with drugs for 24 hours.

Accession Number

Expression and methylation profiling data from DLBCL cell lines have been deposited in the Gene Expression Omnibus and are accessible through accession number GSE28744.

Disclosure of Potential Conflicts of Interest

R.L. Elstrom is employed (other than primary affiliation; e.g., as Medical Director at Genentech, Inc., has received commercial research grants from Celgene and GlaxoSmithKline, has received honoraria from the speakers’ bureaus of Seattle Genetics and Genentech, Inc., and is a consultant/advisory board member of Seattle Genetics. P. Martin and J.P. Leonard are consultan/advisory board members of Celgene. A. Melnick has honoraria from the speakers’ bureau of Genentech and is a consultant/advisory board member of Celgene and Bio-Reference Laboratories. No potential conflicts of interest were disclosed by the other authors.

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