Combined DNA Methyltransferase and Histone Deacetylase Inhibition in the Treatment of Myeloid Neoplasms

Steven D. Gore,1 Stephen Baylin,1 Elizabeth Sugar,1 Hetty Carraway,1 Carole B. Miller,1 Michael Carducci,1 Michael Grever,2 Oliver Galm,1 Tianna Dauses,1 Judith E. Karp,1 Michelle A. Rudek,1 Ming Zhao,1 B. Douglas Smith,1 Jasper Manning,1 Anchalee Jiemjit,1 George Dover,2 Abbie Mays,1 James Zwiebel,4 Anthony Murgo,4 Li-Jun Weng,1 and James G. Herman1

1The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland; 2James Cancer Center of Ohio State University, Columbus, Ohio; Medizinische Klinik IV, Universitätsklinikum Aachen, Rheinisch-Westfälische Technische Hochschule Aachen, Aachen, Germany; and 4Cancer Therapy Evaluation Program, National Cancer Institute, Bethesda, Maryland

Abstract

Optimal reexpression of most genes silenced through promoter methylation requires the sequential application of DNA methyltransferase inhibitors followed by histone deacetylase inhibitors in tumor cell cultures. Patients with myelodysplastic syndrome or acute myeloid leukemia (AML) were treated with the methyltransferase inhibitor 5-azacitidine (aza-CR) followed by the histone deacetylase inhibitor sodium phenylbutyrate. Major responses associated with cytogenetic complete response developed in patients receiving prolonged dosing schedules of aza-CR. Bisulfite sequencing of the p15 promoter in marrow DNA during the first cycle of treatment showed heterogeneous allelic demethylation in three responding patients, suggesting ongoing demethylation within the tumor clone, but no demethylation in two nonresponders. Six of six responding patients with pretreatment methylation of p15 or CDH-1 promoters reversed methylation during the first cycle of therapy (methylation-specific PCR), whereas none of six nonresponders showed any demethylation. Gene demethylation correlated with the area under the aza-CR plasma concentration-time curve. Administration of both drugs was associated with induction of acetylation of histones H3 and H4. This study provides the first demonstration that molecular mechanisms responsible for responses to DNA methyltransferase/histone deacetylase inhibitor combinations may include reversal of aberrant epigenetic gene silencing. The promising percentage of major hematologic responses justifies the testing of such combinations in prospective randomized trials. (Cancer Res 2006; 66(12): 6361-9)

Introduction

The malignant phenotype results from a combination of genetic abnormalities and epigenetic modifications, leading to dysregulation of critical genes controlling cell proliferation, differentiation, and death. Considerable data highlight the important role that methylation of cytosine residues in promoter regions plays in the transcriptional silencing of genes (1, 2). Methylated CpG dinucleotides bind specific proteins, such as MeCP2, which recruit transcriptional corepressors (3). These transcriptional inhibitory complexes include histone deacetylases (HDAC). DNA methyltransferases (DNMT), which catalyze DNA methylation, also bind to HDACs and have the potential to target these enzymes to regions of gene silencing (1, 4–7). Removal of acetyl groups from histone lysine tails is but one of several modifications made to these proteins now known to associate with transcriptional silencing (8, 9). In fact, aberrantly hypermethylated and silenced genes in cancer are now known to have key histone modifications associated with their promoter regions (10–13). However, in the context of all of these layered silencing chromatin modifications that affect such genes, DNA methylation seems to be dominant in specifying tight heritable transcriptional repression. Thus, in cultures of cancer cells, the administration of HDAC inhibitors will not result in reexpression of densely hypermethylated genes until a DNMT inhibitor is first given—the two inhibitors are then synergistic for reexpression of the genes (14, 15).

Given the above dynamics underlying the silencing of genes with hypermethylated promoters, the sequential application of HDAC inhibitors following DNMT inhibitors could potentially increase the response rate, response duration, or percentage of complete responses (CR) in myeloid leukemias through the reexpression of silenced important cell regulatory genes. Multiple such genes are known to exist in hematopoietic neoplasms, including the key cell cycle regulating gene p15 (16–19).

The marked clinical activity of the DNMT inhibitors 5-azacitidine (aza-CR) and 2′-deoxy-5-azacytidine (DAC) in patients with myelodysplastic syndromes (MDS) suggest that epigenetic silencing of cell regulatory genes may play important roles in the pathophysiology of this group of disorders. In addition to hematologic response rates ranging from 30% to 60% (20–23), both drugs have been shown to retard the progression of MDS to acute myeloid leukemia (AML; refs. 23, 24).

The presently described clinical trial was designed to determine whether the sequential administration of a DNMT inhibitor followed by an HDAC inhibitor could be tolerated by patients with myeloid malignancies and to explore the possibility that such combinations would increase the response rate or the quality of responses following aza-CR administration. We combined this drug with the first HDAC inhibitor available for clinical use previously studied by our group (25, 26), sodium phenylbutyrate. This short-chain fatty acid has been successfully used to induce the production of fetal hemoglobin in patients with sickle cell anemia and β-thalassemia (27, 28). In phase I studies in patients with AML...
and MDS, steady-state plasma concentrations at the maximum tolerated dose ranged between 0.3 and 0.5 mmol/L (25, 26), well within the range that induces histone acetylation in vitro (29).

In this first exploration of the feasibility of combined DNMT and HDAC inhibition for the treatment of myeloid malignancies, we sequenced these drugs in a dose-finding study. Modeling the study after in vitro models, patients were treated with the DNMT inhibitor azac-CR followed by the HDAC inhibitor phenylbutyrate. In addition to clinical toxicity and response end points, this study aimed to determine to what extent azac-CR administration was associated with reversal of promotor methylation and whether clinical administration of phenylbutyrate was associated with induction of histone acetylation.

Materials and Methods

Patient Selection

The French-American-British categorization was used to classify patients with MDS and AML; when appropriate, the WHO category of refractory cytopenias with multilineage dysplasia (RCMD) was applied. AML arising with MDS and AML; when appropriate, the WHO category of refractory cytopenia with excess blasts (RAEB) or chronic myelomonocytic leukemia (CMMoL). Patients with RA or RA with ringed sideroblasts were eligible if they met one of several criteria for significant hematologic compromise: absolute neutrophil count <1,000/μL, anemia (RA) with excess blasts (RAEB) or chronic myelomonocytic leukemia (CMMoL). Patients with RAEB-t, low-risk chromosomes (normalities of chromosome 5 or 7, or a normal karyotype), high-risk chromosomes (abnormalities of chromosome 5 or 7, or complex cytogenetic abnormalities), or medical conditions that would compromise the ability to give cytotoxic chemotherapy as the primary treatment modality. Additional eligibility requirements for both groups included Zubrod performance status ≤2, serum creatinine and bilirubin <2.0 mg/dL, and absence of active infections. Administration of hematopoietic growth factors was not allowed during this trial; all growth factor administration was discontinued at least 2 weeks before protocol entry. All patients signed Institutional Review Board–approved informed consent according to Department of Health and Human Services guidelines; additional specific informed consent was obtained for the laboratory research use of patient specimens. Patient characteristics are listed in Table 1.

Treatment Plan

Aza-CR and phenylbutyrate were supplied through the Cancer Therapy Evaluation Program of the National Cancer Institute. All patients received aza-CR s.c. for 5, 10, or 14 days followed by phenylbutyrate given i.v. by continuous 7-day infusion. Aza-CR was supplied as 100 mg of white, lyophilized powder with 100 mg mannitol, USP in 30 mL flint vials. The contents of each vial were dissolved in 4 mL sterile water or 0.9% sodium chloride to provide a 25 mg/mL slurry. Doses were divided so that no single injection constituted >2 mL. Patients were instructed in reconstitution and rapid self-injection of aza-CR. Phenylbutyrate was administered via continuous infusion pump (CADD Legacy Plus ambulatory infusion pump Model 6500, SIMS Deltec, St. Paul, MN). Phenylbutyrate was supplied as a lyophilized powder with 100 mg mannitol, USP in 30 mL flint vials. The clinical administration of phenylbutyrate was associated with induction of histone acetylation.
<table>
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<th>Dose cohort</th>
<th>Cytogenetics</th>
<th>International Prognostic Score</th>
<th>Previously treated</th>
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<th>Response</th>
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<td>M</td>
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<td>Growth factors</td>
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<td>Growth factors</td>
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NOTE: WNL indicates normal cytogenetic analysis. Patients who consented to study but were screen failures or withdrew consent before treatment are not displayed. Four screen failures were not included. Abbreviations: NR, no response; HI-Nmaj, hematologic improvement neutrophils, major; HI-Pmaj, hematologic improvement, platelet, major; HI-Emin, hematologic improvement, erythroid, major.
Aza-CR pharmacokinetics. Samples were obtained before treatment and at 0.25, 0.5, 1, 2, 4, 8, and 24 hours after aza-CR administration. Samples were processed and frozen within 30 minutes by centrifugation at 3,000 \( \times g \) for 5 minutes in a refrigerated centrifuge. Plasma was divided into multiple aliquots to avoid freeze/thaw cycling and was stored at \(-80^\circ C\) until analysis. Initially, samples were collected without tetrahydrouridine. Following observation of samples in which aza-CR could not be detected, presumably due to presumed breakdown by cytidine deaminase, tetrahydrouridine was added to the plasma supernatant at a final concentration of 100 \( \mu \text{mol/L} \) to increase the stability of drug in plasma during storage in the freezer. Tetrahydrouridine increased freezer stability from 7 to 21 days when frozen at \(-80^\circ C\) (35, 36). Aza-CR was quantified in plasma using a previously described validated liquid chromatography/tandem mass spectrometry method over the range of 5 to 300 ng/mL (20.4 to 2,041.6 nmol/L; ref. 36).

Individual concentration-time data were analyzed using noncompartmental methods using WinNonlin Professional version 3.1 as reported elsewhere (Pharsight, Mountain View, CA; ref. 35). Pharmacokinetic variables were summarized using descriptive statistics.

Phenylbutyrate pharmacokinetics. Samples were obtained before treatment and on days 4 and 7 of phenylbutyrate infusion. Samples were collected in heparinized tubes (5 mL) and centrifuged at 3,000 rpm for 5 minutes; plasma was aliquoted and stored at \(-80^\circ C\) until analysis. Plasma was divided into multiple aliquots to avoid freeze/thaw cycling and was stored at \(-80^\circ C\) until analysis. Initially, samples were collected without tetrahydrouridine. Following observation of samples in which phenylbutyrate could not be detected, presumably due to presumed breakdown by cytidine deaminase, tetrahydrouridine was added to the plasma supernatant at a final concentration of 100 \( \mu \text{mol/L} \) to increase the stability of drug in plasma during storage in the freezer. Tetrahydrouridine increased freezer stability from 7 to 21 days when frozen at \(-80^\circ C\) (35, 36). Phenylbutyrate was quantified in plasma using a previously described validated liquid chromatography/tandem mass spectrometry method over the range of 66 to 10,000 ng/mL (20.4 to 3,041.6 nmol/L; ref. 36).

Changes in biological variables over time were represented graphically with additional tests for associations. For each dose group, the association between the mean percentage of F cells and time was tested using a one-way ANOVA. ANOVA was used to determine the association between aza-CR or phenylbutyrate exposure and clinical response as a categorical variable [i.e., 1, a major response (CR or partial response, PR); 2, minor response (i.e., hematologic improvement/lineage response); 3, no response]. A paired \( t \) test was used to determine the association between aza-CR or phenylbutyrate exposure and methylation changes, methylation reversal–induced F cells, and acetylation changes expressed as a categorical variable (i.e., response or no response). The aza-CR exposure variables explored were maximum plasma concentrations (\( C_{\text{max}} \)), area under the concentration-time curve (AUC) from time 0 to 2 hours (AUC(0-2)), AUC from time 0 to 4 hours (AUC(0-4)), AUC from time 0 to the last quantifiable time point (AUC(0-\( t \))), and AUC extrapolated to infinity (AUC(\( t \)). The phenylbutyrate exposure variables explored were \( C_{\text{max}} \) for phenylbutyrate, phenylacetylglutamine, and phenylacetic acid. Statistical analysis was done using JMP Statistical Discovery Software version 3.2.6 (SAS Institute, Cary, NC). The \( a \text{ priori} \) level of significance was \( P < 0.05 \).

Table 2. Accrual to dose-finding trial of aza-CR plus phenylbutyrate in MDS and AML

<table>
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<th>Cohort</th>
<th>Aza-CR dose (mg/m²/d)</th>
<th>Days of aza-CR</th>
<th>Patients assigned to cohort</th>
<th>Number not evaluable</th>
<th>Number reduced to this cohort</th>
<th>Number increased to this cohort</th>
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<td>11/5</td>
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</table>

*For patients who underwent dose escalation or dose reduction, responses are tallied in their final dose cohort.
†Patient originally in dose cohort 4, reduced to dose cohort 3, then reduced to cohort 1.
‡Patient reduced from dose cohort 1.
§Both patients reduced from dose cohort 4; one of these patients was subsequently further reduced to dose cohort 1.
¶Patient dose increased from cohort 5 following lack of response after four cycles.

Results

Study Population

Thirty-six patients signed consent; four patients were screen failures. Characteristics of treated patients are listed in Table 1. Median age was 66 years (41-85 years). Thirteen patients had MDS (including two treatment-induced patients), 1 CMMoL, 15 AML-TLD (MDS-AML), and 3 relapsed AML. Ten of fourteen (71%, 95% confidence interval, 41-92) patients with MDS or CMMoL and 14 of 18 (78%, 95% confidence interval, 52-94) patients with AML had clonal cytogenetic abnormalities. International Prognostic Score categories among MDS/CMMoL patients were as follows: low, 1; Int-1, 7; Int-2, 4; high, 2 (40).

Toxicity

No unusual toxicities were discovered in this trial. Three patients withdrew consent before completing a single cycle: one because of needle phobia, one because of poor performance status, and one for undisclosed personal reasons. Mild nausea was frequent in patients receiving aza-CR and occasional in patients receiving phenylbutyrate; nausea was alleviated in all cases with either 10 mg prochlorperazine or 100 mg dolasetron. Injection site reactions were common in response to aza-CR. One patient in dose cohort 1 developed severe asthenia requiring a dose reduction of aza-CR to 50 mg/m²/d × 5 days. Encephalopathy attributed to phenylbutyrate occurred in nine patients. As in the previous phase I studies of phenylbutyrate, encephalopathy completely reversed within 24 to 48 hours of stopping the infusion. In four cases,
encephalopathy was mild and the 7-day infusion could be successfully completed. Three patients received dose reductions of phenylbutyrate in subsequent treatment cycles (325 mg/kg/d). Two patients tolerated the lower dose; one patient developed severe recurrent encephalopathy and was removed from the trial following his third cycle. The final patient decided not to resume treatment upon recovery from initial symptoms of encephalopathy.

Dose-limiting hematologic toxicity was observed in dose cohort 4 (50 mg/m²/d × 14 days). These toxicities include dose-limiting neutropenia (one patient), dose-limiting thrombocytopenia (two patients), and septic death during neutropenia in cycle 2 (one patient). One surviving patient tolerated dose reduction to 50 mg/m²/d × 10 days; the other patient had recurrent dose-limiting neutropenia at both 50 mg/m²/d (14 days) and 75 mg/m²/d × 5 days and was removed from study. No dose-limiting toxicities occurred in dose cohort 5.

**Clinical Outcomes**
Three patients received less than one complete cycle of therapy and are not considered evaluable for response. Overall, 11 of 29 evaluable patients responded, including 5 of 9 evaluable patients in dose cohort 3 (one patient reduced from dose cohort 4) and 3 of 6 evaluable patients in dose cohort 5. Five were major responders (four CR/one PR); these responders were all treated in dose cohorts 3 (four patients) and 5 (one patient). Seven of 11 responders, including three of the major responders, had clonal cytogenetic abnormalities before treatment. The three major responders had complete cytogenetic responses; none of the minor responders developed cytogenetic remission. Major responses developed in two patients with AML-TLD, one patient with RAEB, one patient with RA with steroid-refractory MDS-associated vasculitis (complete hematologic and rheumatologic response), and one patient with RCMD (complete hematologic response with evidence of ongoing dysplasia in bone marrow). Response duration among major responders was 8, 10, 15, and 19+ months. The patient considered to have “progressed” at 8 months showed cytologic evidence of recurrent MDS with recurrent neutropenia following AML-TLD; complete cytogenetic response persisted.

Minor responses (IWG hematologic improvement-major) occurred in four patients with RAEB (including a patient with therapy-related MDS) and two patients with AML-TLD including four monolineage and two bilineage response (Table 2). Median duration of response among minor responders was 3 months; four patients were removed from protocol with ongoing response because of persistent cytopenias in other lineages or persistent increases in blasts. Among patients who achieved major hematologic improvement, the median number of treatment cycles to observe response (range) were as follows: platelets 1.5 (1-4, n = 7); reticulocytes 3 (2-3, n = 6); hemoglobin 3 (2-4, n = 6); neutrophils 1 (1-5, n = 7). No trend was observed in time to response according to the aza-CR dose cohort.

**Changes in Promoter Methylation**
DNA methylation in bone marrow cells was examined during the first course of drug administration in dose cohorts 3 to 5 to monitor changes within tumor clones rather than monitoring alterations stemming from replacement of neoplastic cells by normal cells. Genomic bisulfite sequencing of a hypermethylated p15 promoter was done in five patients (Fig. 1). Sequential sequencing of DNA from patients who had significant responses to the combined therapy, patients 13 (PR, cohort 3), 19 (CR, cohort 3), and 24 (hematologic improvement, cohort 4, reduced to dose cohort 3) showed significant decrement in p15 methylation following aza-CR treatment. Importantly, we saw a pattern of heterogeneous loss of CpG methylation within each single allele examined, strongly supporting demethylation within the tumor clone. The bisulfite sequencing of patient 19 showed evidence of remethylation before initiation of the next drug cycle. No demethylation was observed in the p15 promoter in two clinical nonresponders (patient 15, cohort 3; patient 34, cohort 5).

Twelve patients evaluable for clinical response were subsequently studied by MSP, a sensitive PCR assay for the methylation of multiple CpG sites in a CpG island (33) for p15 and/or CDH-1. The primers completely overlap the region of the p15 promoter sequenced in the above studies and a region of the CDH-1 gene, which we have used in multiple previous studies. All 12 patients were informative for pretreatment hypermethylation of either or both genes. Six patients developed reversal of methylation for either or both genes during the first cycle of treatment. The methylation status over time of the two genes changed in parallel in three of four patients informative at both loci (Fig. 2A). Particularly noteworthy is patient 19: No evidence of methylation of either gene was present following aza-CR (day 10); however, some evidence of remethylation of both genes was present following phenylbutyrate (day 17). Most importantly, these six patients with reversed methylation of either gene all developed hematologic responses, including three CR patients, one PR, and two patients who achieved hematologic improvement. In contrast, the six patients who did not show methylation reversal were all non-responders. The difference in response rates between the two groups was statistically significant (P = 0.002, Fisher’s exact test).

**Histone acetylation.** Changes in histone acetylation in peripheral blood and bone marrow mononuclear cells were monitored using Western analysis. A representative example is displayed in Fig. 3A. Eleven of 23 evaluable patients showed increases (minimum 50%) in global acetylation of H3 and/or H4 in response to aza-CR alone, similar to the example shown in Fig. 3A. This was observed in one of five patients in dose cohort 1, three of four in dose cohort 2, five of seven in cohort 3, and one of three each in cohorts 4 and 5 (P = not significant between cohorts). Further increases in acetylation in response to phenylbutyrate (minimum 50%) were found in 12 of 23 patients. Overall, 17 of 23 patients developed increased histone acetylation following either aza-CR or phenylbutyrate. Figure 3B displays the changes in histone acetylation from patients in dose cohort 3 semiquantitatively. Blots were stripped and reprobed with antibody to nonacetylated histone H2A. Gels were scanned and band intensity was quantified. The data were then reported as an acetylation index: band intensity acetylated H3 or H4/band intensity nonacetylated H2A. The greatest increment in acetylation appeared following aza-CR administration; phenylbutyrate induced a smaller increment in histone acetylation in many patients. Acetylation remained elevated compared with baseline during phenylbutyrate infusions even in patients who showed no further increment in acetylation in response to phenylbutyrate.

**F cells.** Changes in percentage of RBC containing hemoglobin F (F cells) were monitored as an indirect measure of induction of expression of a surrogate gene silenced through epigenetic mechanisms (γ-globin). Baseline F-cell data were obtained on 26 patients who consented to study. The mean percentage F cells was 7.9% (median 4.9%, range 0.9-48.3%). There was no statistical difference between the mean percentage F cells of patients with the diagnoses of MDS or AML nor was there a statistical difference in the proportion of patients with each diagnosis in patients whose F cells were above or below the mean.
Sequential F-cell data were obtained on 24 patients (Fig. 4). Six patients increased percentage of F cells over time. Three patterns of F-cell induction were observed: a gradual induction throughout the first cycle of therapy (three patients, all in dose cohort 3); delayed induction, coincident with clinical response (one patient in dose cohort 3 and one in dose cohort 5); and transient increase in F-cell production in response to phenylbutyrate (one patient in dose cohort 2). Each cohort was examined for a correlation between time point and F-cell percentage by ANOVA. An association between F cell and treatment time, indicating an increase in F cells over time, was found only dose cohort 3. Three of four clinical responders in cohort 3 who were evaluated for F-cell response developed significant increases in F cells. The other patient in this cohort with an F-cell response did not respond clinically. The patients in cohorts 2 and 5 who developed significant increases in F cells were clinical non-responders. No significant correlation between changes in F cells and clinical response was found when all evaluable patients were examined.

**Aza-CR pharmacokinetics.** The results of the aza-CR pharmacokinetics, combined with results from a trial in nonhematologic tumors, have been reported elsewhere (35). The current analysis includes data beginning with patient 18; tetrahydrouridine, required for stability of aza-CR, was added to the plasma starting with this patient. Aza-CR was rapidly absorbed with the mean $T_{\text{max}}$ occurring at 0.50 hour. Average maximum concentration ($C_{\text{max}}$) and area under the curve (AUC$_{0-\infty}$) values increased in a dose-proportionate manner with increasing dose from 25 to 75 mg/m$^2$/d; the mean ± SD $C_{\text{max}}$ and AUC$_{0-\infty}$ at 25 mg/m$^2$/d were 1,612 ± 1,042 nmol/L and 1,941 ± 980 h × nmol/L, respectively, and at 75 mg/m$^2$/d was 6,207 nmol/L and 5,831 h × nmol/L, respectively. Aza-CR has a short terminal half-life of 1.04 ± 0.88 hours.

A trend to correlation between aza-CR AUC$_{0-\infty}$ and reversal of methylation was detected among the small number of patients.
with complete methylation and pharmacokinetic data ($n = 9$, $P = 0.0658$; Fig. 2B). Aza-CR exposure was not correlated with clinical response, changes in histone acetylation, or induced F cells.

**Phenylbutyrate pharmacokinetics.** The median steady-state phenylbutyrate plasma concentration was 322.5 $\mu$mol/L (mean $562.7 \pm 818.9$, range 19.5-4,371.2). For phenylacetic acid, the median steady-state plasma concentration was 723.9 $\mu$mol/L (mean $1,617.1 \pm 198.7$, range 250.2-6,769.2). For phenylacetylglutamine, the median steady-state plasma concentration was 440.3 $\mu$mol/L (mean $677.6 \pm 457.1$, range 153.4-1,520.9). A total of six patients displayed an unidentified phenylbutyrate metabolite in the plasma samples, which was quantified from the phenylbutyrate standard curve. For the unknown metabolite, the median steady-state plasma concentration was 187.1 $\mu$mol/L (mean 192.8 $\pm$ 71.6, range 84.6-292.9). The median steady-state plasma concentration of patients who did not increase acetylation during phenylbutyrate was 237 $\mu$mol/L (mean 317.1 $\pm$ 100.3). The median steady-state plasma concentration of patients who did not increase acetylation during phenylbutyrate was 343.4 $\mu$mol/L (mean 976.4 $\pm$ 457.5). Phenylbutyrate and metabolite exposure was not correlated with clinical response, changes in histone acetylation, induced F cells, or reversal of methylation.

### Discussion

The DNMT inhibitors aza-CR and DAC comprise two of the most active agents for the treatment of MDS. Despite data demonstrating an ~50% hematologic response rate (23, 41, 42), these agents were developed empirically without careful dose finding, and the molecular mechanism underlying their clinical activity remains unclear. Issa et al. (22) studied DAC administered from 5 to 20 mg/m$^2$/d i.v. for 10 to 20 doses in patients with a variety of hematologic malignancies. The data suggested a potentially improved response rate (65%) in the patients treated at 15 mg/m$^2$/d for 10 doses. Responses were not correlated with baseline p15 methylation or changes in p15 methylation in peripheral blood mononuclear cells during treatment (22).

Daskalakis et al. (43) monitored p15 methylation during treatment of MDS patients with a more dose-dense schedule of DAC (45 mg/m$^2$/d $\times$ 3 days). This group detected decreased methylation in 9 of 12 patients studied investigated at a median time following 3.5 cycles of DAC (range 1-6 cycles). Mund et al. (44) showed decreased methylation in three of four MDS patients treated with DAC, determined following the first cycle of DAC in only two patients. Significantly decreased methylation could be detected after cycle 2 in one patient, cycle 4 in a second patient, and cycle 5 in the third patient. Karyotype normalization preceded changes in genomic methylation; thus, the methylation changes measured likely occurred in normal cells and were irrelevant to proximal molecular changes induced by DAC.

The current study is the first to perform bisulfite sequencing of hypermethylated gene promoters in bone marrow cells during the first cycle of aza-CR. Immediately following the first 10- to 14-day exposure to aza-CR in three clinically responding patients, bone marrow cells possess heterogeneously methylated alleles and an incomplete demethylation pattern. This observation confirms that changes in methylation detected following aza-CR do not arise from the replacement of the abnormal (methylated) clone with normal (unmethylated) cells following a cytotoxic event. The precise parallels between the bisulfite sequencing data and MSP results allowed us to use the latter procedure to monitor methylation of two gene promoters in clinical samples. The results, requiring confirmation in a larger series, revealed a tight correlation between initial tumor clone changes in hypermethylation of the p15 and CDH-1 promoters and clinical response. The development of cytogenetic complete remission in a patient in whom partial demethylation developed in malignant cells during cycle 1 suggests that these agents, which target epigenetically silenced transcription, may activate genetic programs leading to terminal differentiation, apoptosis, or senescence. More normal clones can then replace the starting population. Partial demethylation fits well with the possibility that HDAC inhibitors may increase therapeutic efficacy by contributing to reexpression of...

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**Figure 2.** Changes in methylation of p15 and CDH-1 promoters during cycle 1 of treatment. A, bone marrow mononuclear cells were procured before therapy (1), following the last injection of aza-CR (2), following discontinuation of phenylbutyrate (3), and before cycle 2 (4). In one patient, a fifth time point was procured after four cycles of treatment. Methylation of p15 and CDH-1 promoters was determined using MSP. The four representative patients were all treated in dose cohort 3 (50 mg/m$^2$/d aza-CR for 10 days). NI, normal peripheral blood lymphocytes. IVD, positive methylation control. B, reversal of promoter methylation for either p15 and or CDH-1 after one cycle of therapy is plotted against the aza-CR AUC for the first dose of drug for nine patients with complete set of methylation and pharmacokinetic data. Correlation approached statistical significance ($P = 0.0658$). Dotted line, median concentration. Solid line, mean concentration.
densely hypermethylated genes once some demethylation is initiated by drugs, such as aza-CR, as shown in vitro (14, 15).

The pharmacokinetic/pharmacodynamic studies suggest that reversal of promoter methylation may correlate with AUC for aza-CR. Nonresponse may be due to failure of adequate exposure of the target to drug, raising hopes that adjusted dosing schedules might improve clinical outcomes further. The relationship between aza-CR AUC and methylation reversal could be due to genetic polymorphisms affecting drug metabolism; one candidate would be differences in cytidine deaminase, the primary catabolic enzyme for aza-CR. Polymorphisms and differences in expression of cytidine deaminase and other catabolizing enzymes have been associated with in vivo resistance to cytarabine (45–48). These data suggest that more easily administered formulations of aza-CR could be developed, with potential for an adaptive control mechanism to increase the percentage of patients with promoter methylation reversal.

The current study design does not allow assessment of the specific biochemical contribution of the HDAC inhibitor. Ultimately, a randomized trial of a DNMT inhibitor with and without an HDAC inhibitor will be required. Increases in global histone acetylation were observed in the majority of patients studied. The induction of histone acetylation in response to aza-CR before the HDAC inhibitor was unexpected. The mechanism by which aza-CR led to histone acetylation is currently unknown; it is not clear whether this is a DNMT-dependent phenomenon and whether similar induction of histone acetylation occurs in response to in vivo administration of DAC. Increases in acetylation could be specifically attributed to phenylbutyrate in only 12 of 23 patients. This may be due to maximal induction of acetylation in response to aza-CR alone. The median steady-state plasma concentration of phenylbutyrate was consistent with previous phase I studies and would be predicted to be adequate for the induction of acetylation (25, 26, 37–39). However, the large percentage of patients evaluable for acetylation who developed increments in this endpoint (17 of 23) precludes assessment of the contribution of HDAC inhibition to clinical response.

From a clinical perspective, larger trials will be required to assess the efficacy of combining aza-CR with HDAC inhibitors in the treatment of MDS and AML. The current trial shows the clinical feasibility of combining a DNMT and HDAC inhibitor. Our current study also suggests that aza-CR in combination with phenylbutyrate yields a potential enrichment in major responses in patients who received aza-CR at 50 mg/m²/d × 10 days followed by phenylbutyrate. In the definitive Cancer and Leukemia Group B study of aza-CR, the CR plus PR rate was 23% (23). In dose cohort 3, four of nine evaluable patients achieved CR or PR. These numbers are small and require confirmation in a phase II setting; however, they suggest the possibility that prolonged exposure to lower doses of a DNMT inhibitor and/or the addition of an HDAC inhibitor may increase the major response rate.

Figure 3. Changes in global histone acetylation during cycle 1 of therapy. Histones were isolated from peripheral blood and bone marrow mononuclear cells during cycle 1 of therapy. Immunoblotting was done using antibodies to acetylated H3 and H4. Blots were then stripped and reprobed using antibody against nonacetylated histone H2A as a control for the efficiency of histone extraction and protein loading. A, representative patient (patient 14, cohort 3, AML-TLD, nonresponder). Pre, pretreatment. D, day of treatment. Blotting of H2A is done following stripping of the membrane after blotting for acetylated H3 and H4. Some residual Ac-H3 and Ac-H4 continues to be visualized on the H2A blot.

Figure 4. Changes in F cells over time. The percentage of F cells in patients’ peripheral blood was monitored using flow cytometry before treatment, after aza-CR, after phenylbutyrate, before cycle 2, and at later time points (either before cycle 3 or 4). The patient data are displayed by dose cohort. In patients in whom a value is missing, the trend lines are discontinuous. Arrows, patients with significant increase.
In summary, we have developed a well-tolerated combination schedule of azac-Ar and HDAC inhibitor, which induces promoter methylation reversal, global histone acetylation, F-cell production, and clinical responses. This first study provides a recommended phase II dose schedule for azac-Ar (50 mg/m^2/d × 10 days s.c.) in studies involving comparisons without an HDAC inhibitor to fully evaluate the efficacy of combining such drugs. We have also shown the first solid evidence that demethylation of hypermethylated genes in tumor clones is a target effect of azac-Ar in patients who respond to therapy with this drug. This provides a possible assay to make early judgments about efficacy in individual patients and may be evidence that demethylation may underlie the responses themselves.

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Steven D. Gore, Stephen Baylin, Elizabeth Sugar, et al.


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