A Phase I Dose-Finding Study of 5-Azacytidine in Combination with Sodium Phenylbutyrate in Patients with Refractory Solid Tumors

Jianqing Lin,[1] Jill Gilbert,[4] Michelle A. Rudek,[1] James A. Zwiebel,[6] Steve Gore,[3] Anchalee Jiemjit,[1] Ming Zhao,[1] Sharyn D. Baker,[5] Richard F. Ambinder,[3] James G. Herman,[2] Ross C. Donehower,[1] and Michael A. Carducci[1]

Abstract

Purpose: This was a phase I trial to determine the minimal effective dose and optimal dose schedule for 5-azacytidine (5-AC) in combination with sodium phenylbutyrate in patients with refractory solid tumors. The pharmacokinetics, pharmacodynamics, and antineoplastic effects were also studied.

Experimental Design: Three dosing regimens were studied in 27 patients with advanced solid tumors, and toxicity was recorded. The pharmacokinetics of the combination of drugs was evaluated. Repeat tumor biopsies and peripheral blood mononuclear cells (PBMC) were analyzed to evaluate epigenetic changes in response to therapy. EBV titers were evaluated as a surrogate measure for gene re-expression of epigenetic modulation in PBMC.

Results: The three dose regimens of 5-AC and phenylbutyrate were generally well tolerated and safe. A total of 48 cycles was administrated to 27 patients. The most common toxicities were bone marrow suppression–related neutropenia and anemia, which were minor. The clinical response rate was disappointing for the combination of agents. One patient showed stable disease for 5 months whereas 26 patients showed progressive disease as the best tumor response. The administration of phenylbutyrate and 5-AC did not seem to alter the pharmacokinetics of either drug. Although there were individual cases of targeted DNA methyltransferase activity and histone H3/4 acetylation changes from paired biopsy or PBMC, no conclusive statement can be made based on these limited correlative studies.

Conclusion: The combination of 5-AC and phenylbutyrate across three dose schedules was generally well tolerated and safe, yet lacked any real evidence for clinical benefit. (Clin Cancer Res 2009;15(19):6241–9)

Authors' Affiliations: [1]Chemical Therapeutics Program, [2]Cancer Biology, and [3]Hematologic Malignancies, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Johns Hopkins University, Baltimore, Maryland; [4]Department of Medical Oncology, The Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, Tennessee; [5]St. Jude Children's Hospital, Memphis, Tennessee; and [6]Cancer Therapy and Evaluation Program, National Cancer Institute, NIH, Bethesda, Maryland

Received 3/9/09; revised 6/10/09; accepted 7/1/09; published OnlineFirst 9/29/09.

Grant support: NCI UO1-CA70095 (R.C. Donehower and M.A. Carducci), NCI P50-CA58236 (J.G. Herman and M.A. Carducci), NCI P30-CA08973 (M.A. Rudek, M.A. Carducci, and R.F. Ambinder), Prostate Cancer Foundation (M.A. Carducci), AEGON International Fellowship in Oncology (J. Gilbert), and NCI T32 (J. Lin). This study was sponsored by the Cancer Therapy Evaluation Program of the National Cancer Institute, NCI CA Specialized Programs of Research Excellence, and AEGON.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Michael A. Carducci, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, CRBI 1M59, 1650 Orleans Street, Baltimore, MD 21231. Phone: 410-614-3977; Fax: 410-614-8160; E-mail: Carducci@jhmi.edu.

doi:10.1158/1078-0432.CCR-09-0567

Epigenetic silencing of key genes, such as tumor suppressor genes, contribute to carcinogenesis (1–4). Two epigenetic processes, DNA methylation and histone acetylation, result in the silencing of target genes (5). Methylation of the CpG islands of gene promoter regions induces a conformational change in the chromatin, leading to transcriptional silencing. Methylated DNA then recruits and binds methyl-CpG–binding domain proteins, which directly repress transcription and also complex with transcriptional corepressors including histone deacetylases (HDAC; ref. 6). Histone acetylation is associated with an open chromatin conformation, allowing gene transcription. HDACs maintain chromatin in the closed, nontranscribed state. As individual epigenetic modifications, both DNA methylation and histone deacetylation contribute to a "closed" chromatin conformation, inhibiting transcription. However, epigenetic modulation via HDAC inhibition following demethylation could lead to more robust expression of previously silenced genes than inhibition of either process alone. Thus, drugs targeting DNA methylation and histone deacetylation remain an active and promising area of clinical investigation for cancer therapeutics (7–9).

The cytidine analogue, 5-azacytidine (5-AC) inhibits DNA methyltransferases (DNMT), leading to demethylation of

Translational Relevance

Preclinical data shows that epigenetic modulation via histone deacetylase and DNA methyltransferase inhibition could lead to robust expression of previously silenced genes, holding promise as a therapeutic strategy, especially in tumors with silenced transcription of tumor suppressor genes. 5-Azacytidine (5-AC) and phenylbutyrate can individually upregulate the transcription of epigenetically silenced genes. Multiple 5-AC dosing schedules in combination with phenylbutyrate were explored in this study. Furthermore, the study sought to determine whether 5-AC administered at lower doses for longer periods of time would result in consistent biological or clinical responses than shorter duration of 5-AC administration.

DNA in daughter cells with a resultant effect on gene expression and cell differentiation (10). Once incorporated into DNA, 5-AC produces a marked dose-dependent and time-dependent decrease in DNMT activity. Clinical trials of single-agent 5-AC in solid tumor malignancies have been disappointing with little clinical activity (11–14). In these early trials of solid tumor malignancies, methylation status and gene expression were not reported. However, 5-AC showed evidence of transcriptional modulation, global hypomethylation, and clinical response in trials of patients with hemoglobinopathies and myeloid disorders (10, 15–17). 5-AC is Food and Drug Administration–approved for the treatment of myelodysplastic syndrome at a dose of 75 mg/m^2/d for 7 days every 4 weeks (18). At this dose and schedule, cytopenias, nausea, and vomiting proved to be the most common side effects (17).

Phenylbutyrate, a first-generation HDAC inhibitor, is Food and Drug Administration–approved for the treatment of hyperammonemia in patients with urea cycle disorder. Phenylbutyrate results in the acetylation of histones *in vitro*, alters gene expression, and promotes differentiation (19). Two phase I studies of phenylbutyrate in refractory solid tumors have been reported (20, 21). In the study using intravenous administration, the maximum tolerated dose (MTD) was 410 mg/kg/d for 5 days. Toxicity was primarily neurocortical and was readily reversible after discontinuation of the drug (21). Evidence of gene expression modulation was shown.

Because demethylation predominantly occurs in S phase, longer periods of exposure to 5-AC may lead to a greater proportion of cells exposed during S phase, potentially leading to a greater effect on methylation status. In contrast, phenylbutyrate leads to G_1/G_0 growth arrest within 96 hours (22, 23). Maximal re-expression of key genes may require actively dividing cells. Given the epigenetic "layers" of transcriptional silencing and the evidence that demethylation followed by HDAC inhibition results in robust gene expression (7), we hypothesized that maximal gene expression and minimal toxicity would occur with the administration of lower doses of 5-AC for longer periods of time in conjunction with HDAC inhibition. Thus, this study investigated multiple 5-AC dosing schedules in combination with intermittent dosing schedules of phenylbutyrate. The primary objective of this dose-finding study was to determine the minimal effective dose (MED) of the combination of agents that results in clinical response and/or target inhibition. Secondarily, we sought the acute and chronic toxicity profile, and the pharmacokinetics of 5-AC and phenylbutyrate when used in combination. Moreover, as methylation silences incorporated viral genomes, such as EBV (24–27), we studied the EBV viral load changes as a surrogate marker of gene re-expression after treatment with 5-AC and sought to correlate changes with the pharmacokinetics.

Patients and Methods

Patients age 18 or older with tissue or cytologic diagnosis of refractory solid tumor malignancy and no curative options were eligible for the study. Other eligibility criteria included documentation of evaluable tumor, Eastern Cooperative Oncology Group performance status ≤2, life expectancy of 12 wk or longer, and adequate bone marrow, hepatic, and renal function. Patients were required to have a negative serum HIV test and no evidence of central nervous system metastasis (28). Prior chemotherapy or radiation therapy was acceptable if they were completed ≥4 wk prior to entry, with recovery from any toxicities (to grade 1 or grade 0). Patients were required to provide written informed consent prior to study enrollment. The study was approved by the Cancer Review Committee and the Institutional Review Board of the Johns Hopkins University School of Medicine.

Toxicity was classified and graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 2.0. Standard WHO criteria were used to define response or progression. Patients were considered evaluable for toxicity once therapy was initiated. The treatment responses for therapy were evaluated every two cycles. Patients with progressive disease were removed from the study. Patients with complete or partial responses or stable disease continued treatment until evidence of progression, unacceptable toxicity, or desire to withdraw from the study.

Drug administration. 5-AC and phenylbutyrate were supplied by the Cancer Therapy Evaluation Program of the National Cancer Institute. 5-AC was supplied as 100 mg of white, lyophilized powder with 100 mg of mannitol, USP in 30 mL flint vials. The contents of each vial were dissolved in 4 mL of 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. 5-AC was administered subcutaneously within 30 min of reconstitution with daily rotation of the injection site. An administration log that recorded the dates and times of administration was maintained by each patient. When pharmacokinetic studies were done, the 5-AC dose was administered by trained research staff. Phenylbutyrate was supplied as a 400 mg/mL (40%) viscous solution in sterile water with a pH of 7.5 to 9.5 in 50-mL vials. Each milliliter of solution contained 98 mg of sodium. In order to administer this drug as a continuous infusion, the total daily dose was diluted in 1 L of sterile water for injection. Phenylbutyrate was administered as a continuous infusion (CIV) over 24 h and bags were changed daily (21).

The study evaluated three schedules of administration of the combination of agents (please see Table 1). Regimen A evaluated a 14-d low-dose regimen of 5-AC with intermittent phenylbutyrate by CIV over 24 h on days 6 and 13. Regimen B evaluated 5-AC given s.c. for 7 d at a daily dose of 75 mg/m^2/d. 5-AC was followed sequentially by two different doses of CIV phenylbutyrate starting on day 8 and continuing for 7 d. This regimen of 5-AC was chosen due to the fact that the same dose and schedule showed clinical efficacy for the treatment of myelodysplastic syndrome. Regimen C evaluated the lowest daily doses of 5-AC, given for 21 d with phenylbutyrate given as a CIV infusion over 24 h once per week, whereas the patient received 5-AC. Cycle length for regimens A and B was 35 d, and for regimen C was 42 d.

Dose modification. Nonhematologic dose-limiting toxicity (DLT) was defined as National Cancer Institute/Division of Cancer Treatment Common Toxicity Criteria grade 3 or 4 toxicity. Hematologic DLT was defined as grade 3 or 4 neutropenia associated with a fever or lasting 5 d. Anemia was not a DLT as RBC transfusion was allowed. DLT affecting dose escalation was determined during cycle 1. For patients that developed Common Toxicity Criteria grade 3 or 4 toxicity which was judged to be clinically significant by the principal investigators, treatment was withheld until resolution (for no more than 2 wk) of toxicity to grade 2 or less. The treatment was then resumed at a 25% dose reduction. If patients experienced a grade 3 or 4 toxicity at the reduced dose, the treatment was interrupted until the toxicity resolved (for no more than 2 wk) to grade 2 or less. The treatment resumed at a 50% dose reduction of the initial dose. Patients who required more than two dose reductions were removed from the study.

If one out of three patients in a dose level experienced a DLT during cycle 1, then three additional patients were enrolled at that dose level. If zero of three or only one of the six patients in the expanded dose cohort experienced a DLT, then the next dose level was allowed to proceed. If two or more out of six patients experienced a DLT, then the preceding dose level was considered the MTD. The MTD was defined as the highest dose level of 5-AC in combination with phenylbutyrate that caused one or fewer of six patients to experience DLTs. Because of the development of neutropenia side effect from dose level A1, cohorts A-1 and A-2 were explored and the protocol amended such that subsequent cohorts were enrolled in the following sequence: A1 → A-1 → A-2 → B1 → A-3 → B2 → C1 → C2.

The MED that elicited a biological or clinical response was also examined. In contrast to a more traditional MTD, the MED was to be determined by evidence of drug target inhibition or clinical response criteria. The MED was not determined on a real-time basis as the results of the correlative studies lagged behind the accrual needs of the trial.

Pharmacokinetic studies. For 5-AC, samples were obtained prior to treatment and at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after 5-AC. Samples were stored and processed as described (29, 30). Pharmacokinetic variables were determined as previously described (29).

For phenylbutyrate pharmacokinetics, samples were obtained prior to treatment and on the first day of the phenylbutyrate infusion. When phenylbutyrate was administered as a 24-h infusion, additional samples were obtained at 0.5, 1, and 4 h during the infusion; immediately before the end of infusion (24 h); and postinfusion at 0.25, 0.5, 1, 2, 4, 8, and 24 h after the end of the infusion. When phenylbutyrate was administered as a 7-d infusion, additional samples were obtained at 1, 4, 24, and 72 h during the infusion and immediately before the end of infusion (168 h). Samples were analyzed for phenylbutyrate and two metabolites, phenylacetylglutamine and phenylacetic acid, with a high-performance liquid chromatography method using UV detection (20, 21, 31). The linear calibration curves were generated over the range of 10 to 3,000 μmol/L. When phenylbutyrate was administered as a 24-h infusion, individual concentration-time data were analyzed using noncompartmental methods using WinNonlin Professional version 5.0 as described above for 5-AC with the following exceptions: (*a*) maximal plasma concentration (C_{max}) was the concentration obtained prior to the end of the infusion; (*b*) the AUC value was calculated using the linear trapezoidal rule; and (*c*) no weight was used when calculating λ_z. When phenylbutyrate was administered as a 7-d infusion, average steady-state concentrations (C_{ss}) were calculated for phenylbutyrate, phenylacetylglutamine, and phenylacetate based on graphical presentation of concentration-time profiles.

Correlative studies. Biological end points were measured using pretreatment and posttreatment tumor biopsy, and peripheral blood mononuclear cells (PBMC). Biopsy of tumor for research purposes was optional for patient enrollment. Tumor markers such as prostate-specific antigen, CA 19-9, were evaluated and followed where appropriate. The correlative studies included (*a*) the determination of DNMT enzyme activity (32). Briefly, this was measured by incubating 10 μg of tissue protein lysate with 3 μCi of *S*-adenosyl-L-[methyl-^{3}H]methionine and 0.5 μg poly(dI-dC)-(dI-dC) or 2.5 μg of oligonucleotide 5′-CCAGCCCGGCCCGACCCGACCGCACCCGGCGC-3′ (methylated cytosines are underlined) for 120 min at 37°C. Results were expressed as the mean dpm per microgram of protein. Tumor tissues were obtained prior to therapy (within 4 wk of starting therapy) and on day 14 or 21 in cycle 1 after therapy. (*b*) Determination of histone acetylation in PBMC by Western blot using polyclonal antibodies directed against acetylated histones H3 and H4 and nonacetylated H2 (33). PBMCs were collected within 4 wk before treatment as baseline, on treatment day 6 (for dose schedule A and C) or day 7 (schedule B) right before starting phenylbutyrate infusion, and on day 14 (schedule A and C) or day 15 (schedule B) when phenylbutyrate infusion was finished. (*c*) Determination of EBV viral load in PBMC by real-time PCR. PBMC samples from baseline and treatment days 1, 7, and 15 were collected (>3 million cells). Samples were snap-frozen and DNA was extracted for quantitative competitive PCR analysis using specific primers as described previously (34).

Statistical considerations. Pharmacokinetic variables were summarized using descriptive statistics. Graphical presentation of concentration-time profiles consisted of the average and SD of the concentrations determined at each time point. Dose-independent pharmacokinetic variables (T_{max}, $T_{1/2}$, Cl/F, and Vd/F) and dose-normalized dose-dependent pharmacokinetic variables (C_{max}/dose and $AUC_{[INF]}$/dose) were compared using one-way ANOVA to compare the differences as a function of dose levels. Statistical analysis was done using JMP Statistical Discovery Software version 4.04 (SAS Institute). The a priori level of significance was $P < 0.05$. Because a limited number of

Table 1. Schema for regimens

Dose levels	AC, mg/M^2/d (days)	Phenylbutyrate, mg/m^2/d (days)	No. of patients	Days/cycle
A				
A1	25 (D1-14)	400 (D6, D13)	3	35
A-1	18.75 (D1-14)	400 (D6, D13)	3	
A-2	15 (D1-14)	400 (D6, D13)	3	
A-3	10 (D1-14)	400 (D6, D13)	3	
B				
B1	75 (D1-7)	200 (D8-14)	3	
B2	75 (D1-7)	400 (D8-14)	4*	
C				
C1	10 (D1-21)	400 (D6, D13, D20)	3	42
C2	12.5 (D1-21)	400 (D6, D13, D20)	6	

*One patient did not receive phenylbutyrate.

Table 2. Patient characteristics

Characteristics	No. of patients
No. of patients	28
Sex (male/female)	17/11
Age (y), median (range)	59 (34-82)
Race	
Caucasian/Black/Asian	25/2/1
ECOG performance status	
0	10
1	17
2	1
Primary tumor site	
Colorectal	7
Prostate	5
Pancreas (neuroendocrine)	1
Kidney	3
Esophageal	3
Breast	2
Sarcoma	2
Bladder	2
Liver	1
Lung	1
Mesothelia	1
Average prior chemotherapy regimens	2.5

Abbreviation: ECOG-PS, Eastern Cooperative Oncology Group performance status.

samples were available for correlative studies, the analysis was descriptive only.

Results

Patient characteristics. Between March 2000 and July 2005, a total 34 patients with advanced solid tumors were enrolled into the study. Six patients did not meet the eligibility requirements and thus were not treated with the protocol. One patient had rapid clinical progression of disease during 5-AC treatment and did not receive phenylbutyrate. This patient was assessable for safety and toxicity but only 27 patients were assessable for pharmacokinetics and efficacy of the combination. The demographic and clinical characteristics of the subjects are summarized in Table 2.

Toxicity. Treatment was generally well tolerated. A total of 48 cycles of treatments were administrated with an average of 1.7 cycles/patient. The most common grade 3 and grade 4 toxicities were neutropenia, which happened mostly in cycle 1. All cohorts had three patients except cohort B2 and C2, in which four and six patients were enrolled. One patient was replaced in cohort B2 because he had disease progression while on 5-AC treatment but did not receive phenylbutyrate. During the first cycle of dose level C2, one patient had worsening nausea and vomiting and was found to have acute chronic renal failure. The etiology was multifactorial including new urinary tract obstruction on the left, secondary to tumor bulk, dehydration, and fungal urinary tract infection. She required left percutaneous nephrostomy tube placement and exchange of the right side nephroureteral stent. Her treatment continued after creatinine went down to 1.9, but because of her general poor performance status and persistent chronic renal insufficiency, she was taken off the study in the middle of the second cycle. This cycle 1 event of renal failure was considered possibly related to the study drug and the cohort was expanded. Five other patients in the same cohort experienced no renal toxicities. Because of the slow accrual and the availability of more potent HDAC inhibitors, no further dose escalations were done after this event.

Potentially treatment-related grade 3 and 4 nonhematologic toxicities during all cycles of therapy are listed in Table 3. At dose level A1, one of three patients had transient grade 3 hyponatremia. One of three patients showed grade 3 somnolence related to phenylbutyrate at dose level B2 during the first cycle, whereas one of three patients showed grade 3 confusion at dose level C1 of the second cycle, which was considered to be possibly from phenylbutyrate. Both toxicities reflect the neurocortical side effects of phenylbutyrate. These symptoms were transient and resolved promptly when phenylbutyrate was withheld.

Hematologic toxicity was moderate (Table 3). Five patients experienced grade 3 neutropenia lasting for less than 5 days, three of three patients on dose level A1 and two of three patients on dose level B2. Patients received a 25% dose reduction without recurrence of neutropenia. No treatment delays occurred due to neutropenia. No episodes of febrile neutropenia were noted. For dose level A1, the protocol was amended to begin dose de-escalation, therefore MTD was underexplored and not determined for dose schedule A. Two of six patients experienced grade 3 anemia at dose level C2 at cycle 2. Both of the patients required transfusion.

For all grade toxicities, the most common adverse events were nonhematologic and included 5-AC injection site reaction, low-grade nausea, vomiting, fatigue, transaminase elevation, edema, hyperglycemia, hyponatremia, light-headedness, anorexia, and diarrhea (Table 3).

Response evaluation. No clinical responses were noted on any regimen or dose level. One patient with leiomyosarcoma on dose level B1 received four cycles of therapy and showed stable disease for 4.5 months. Twenty-six patients had progressive disease as the best clinical response and were removed from study.

Pharmacokinetics. 5-AC pharmacokinetic data obtained from an additional 12 patients (data not shown) were consistent with our previously published data (29). Phenylbutyrate pharmacokinetic studies were completed in all patients, with 21 patients receiving 24-hour infusions and 6 patients received phenylbutyrate as a 7-day infusion. Pharmacokinetic variables for phenylbutyrate administered as a 24-hour infusion are listed in Table 4. All patients who received the 24-hour infusion received 400 mg/kg/d and there was no statistically significant difference in phenylbutyrate, phenylacetate, or phenylacetylglutamine pharmacokinetic variables across the various dose levels ($P > 0.05$). Therefore, for all analyses, all 24-hour infusion pharmacokinetics variables were treated the same. The mean ± SD C_{max}, $AUC_{[INF]}$, and Vd for phenylbutyrate were 775 ± 467 μmol/L, 17,722 ± 8,345 h*μmol/L, and 14.0 ± 8.0 L, respectively. The phenylbutyrate concentrations were sustained above 500 μmol/L for 16.46 hours on average. For phenylacetate, the mean ± SD C_{max}, $AUC_{[INF]}$, and phenylacetate/phenylbutyrate AUC ratio were 1,395 ± 594 μmol/L, 26,680 ± 11,236 h*μmol/L, and 1.71 ± 0.92, respectively. There was more variability in the phenylacetylglutamine exposure with the mean ± SD C_{max}, $AUC_{[INF]}$, and phenylacetylglutamine/phenylbutyrate AUC ratio being 997 ± 507 μmol/L, 25,609 ± 16,771 h*μmol/L,

and 1.85 ± 1.78, respectively. A total of four patients had an unidentified phenylbutyrate metabolite in the plasma samples, which was quantified from the phenylbutyrate standard curve. For the unknown metabolite, the average C_{max} was 316 μmol/L and occurred at 24.2 hours.

When phenylbutyrate was administered as a 7-day infusion, the mean ± SD steady state phenylbutyrate plasma concentrations were 210 ± 73.8 μmol/L at 200 mg/kg/d and 446 ± 211 μmol/L at 400 mg/kg/d. Only one patient had phenylbutyrate concentrations sustained at ~500 μmol/L. For phenylacetate, the mean ± SD steady state plasma concentrations were 184 ± 85.2 μmol/L and 1,464 ± 1,285 μmol/L at 200 and 400 mg/kg/d, respectively. For phenylacetylglutamine, the median steady state plasma concentrations were 427 ± 103 μmol/L at 200 mg/kg/d and 1,217 ± 244 μmol/L at 400 mg/kg/d. No patient displayed an unidentified phenylbutyrate metabolite in the plasma samples as was observed during the 24-hour infusion.

Correlative studies. DNMT activity was analyzed in five patients, from which paired tumor biopsy tissue were available at baseline and after 5-AC treatment (day 14 or 21 in cycle 1). Table 5 showed the dose level of 5-AC, tumor type, and DNMT activities. DNMT levels varied among solid tumors, and patients with prostate adenocarcinoma had low baseline tumor DNMT activity, resulting in the inability to detect significant enzyme inhibition after treatment with 5-AC. However, at the 5-AC dose of 15 mg/m^2/d for 14 days, a 75%, inhibition of DNMT activity was observed in a patient with hepatocellular carcinoma in which basal DNMT activity was much higher. Additionally, a renal cell carcinoma tumor lacked detectable DNMT activity after 5-AC treatment, although the pretreatment enzyme activity was low but detectable. This data suggests that even at daily doses of 15 mg/m^2, there may be inhibition of DNMT in some patients.

All patients' PBMC samples were collected for histone H3 and H4 acetylation study, but only 13 samples were available for analysis based on a number of factors relating to the quality of the immunoblot (assay not fully worked out, laboratory errors, etc.). Table 6 showed the dose level of phenylbutyrate, tumor type, and changes of histone H3 or H4 acetylation in 13 evaluable Western blots. Eleven of 13 (85%) patients had detectable acetylated forms at baseline (day 0) for both H3 and H4. Two of 13 (15%) had no detectable acetylation for H3 but both were induced to have acetylation after 6 days of treatment with 5-AC only at doses of 75 or 12.5 mg/m^2/d before phenylbutyrate treatment. None of these patients had tissue biopsies and thus DNMT activities were not available. Seven patients (54%) showed increased acetylation after either 24 hours of phenylbutyrate infusion or CIV phenylbutyrate for 7 days (Table 6). At dose schedule B, when phenylbutyrate was infused for 7 days, three of five (60%) patients achieved increased acetylation. Four of eight (50%) patients showed acetylation when phenylbutyrate was infused for just 24 hours.

Table 3. Summary of nonhematologic and hematologic toxicity (all cycles) presenting in two or more patients

Dose levels (patients)	Events	A1 (3)		A-1 (3)		A-2 (3)		A-3 (3)		B1 (3)		B2 (3)		C1 (3)		C2 (6)	
		Any	G3	Any	G3	Any	G3	Any	G3	Any	G3	Any	G3	Any	G3	Any	G3
Nonhematologic																	
Injection site reaction	23	3		3		2		3		3		3		3		3	
Nausea	18	1		3		1		2		3		4		2		2	
Vomiting	17	1		3		1		2		3		4		1		2	1*
Fatigue	17	3		1		1		2		3		3		1		3	
ALT elevation	11	1				3				3		2		2			
Edema	10	1		1		1		2		3		1				1	
Hyperglycemia	7			1						3		1		1		1	
Lightheadedness	6			2				1		1		1		1			
Anorexia	5			1										1		3	
Diarrhea	5					1		1				1				2	
Hyponatremia	5	1	1*	3												1	
Abdominal pain	4			1										1		2	
Body odor	4			1		1		1						1			
Headache	3							1		2							
Somnolence	3					1		1				1	1*				
Alopecia	3			1		1				1							
Flatulence/indigestion	3			1										1		1	
Constipation	2	1										1					
Pruritis	2	1								1							
Skin rash	2							1		1							
Arthralgia	2	1		1													
Hypokalemia	2	1				1											
Hypoalbuminemia	2					1								1			
Confusion	2			1										1	1		
Hematologic																	
Neutropenia	11	3*	3*			1*		1*		1*		2*	2*			2*+1	
Anemia	9	2	1	2		1				1		1				2	1*+1
Thrombocytopenia	6			1		1						2		1		1	

Abbreviation: G3, Common Toxicity Criteria grade 3.
*Neutropenia and other grade 3 toxicities in cycle 1.

Table 4. Pharmacokinetic variables of phenylbutyrate administered as a continuous infusion over 24 h to patients with cancer

	Dose/ regimen	*n*	C_{max} (μmol/L)*	T_{max} (h)†	$AUC_{[INF]}$ (h*μmol/L)*	Cl (L/h)*	T > 500 μmol/L (h)†	Metabolite/ phenylbutyrate AUC ratio*
Phenylbutyrate	A-2	9	714 ± 462	24.09 (23.67-25.00)	16,305 ± 8,417	15.0 ± 10.8	14.35 (0.00-25.66)	NC
Phenylbutyrate	A-3	3	1,004 ± 489	24.38 (24.07-24.82)	21,744 ± 7,653	7.0 ± 3.6	23.15 (18.60-26.07)	NC
Phenylbutyrate	C1	3	956 ± 184	24.06 (24.00-24.12)	22,080 ± 2,598	6.7 ± 1.3	24.10 (23.17-24.68)	NC
Phenylbutyrate	C2	6	662 ± 591	24.25 (23.83-25.33)	15,659 ± 10,453	14.8 ± 8.2	12.45 (0.00-25.82)	NC
Overall			775 ± 467	24.17 (23.67-25.33)	17,722 ± 8,345	12.6 ± 8.9	16.46 (0.00-26.07)	
Phenylacetate	A-2	9	1,159 ± 445	25.16 (24.00-26.95)	22,611 ± 11,613	13.2 ± 10.2	NC	1.39 ± 0.19
Phenylacetate	A-3	3	1,932 ± 472	24.92 (24.25-26.00)	33,398 ± 11,492	4.9 ± 1.2	NC	1.64 ± 0.58
Phenylacetate	C1	3	1,417 ± 300	26.04 (24.12-27.92)	30,544 ± 13,291	6.3 ± 2.4	NC	1.35 ± 0.45
Phenylacetate	C2	6	1,471 ± 540	25.32 (23.83-27.98)	27,492 ± 9,956	7.9 ± 3.4	NC	2.40 ± 1.50
Overall			1,395 ± 504	25.30 (23.83-27.98)	26,680 ± 11,236	9.5 ± 7.6		1.71 ± 0.92
Phenylacetylglutamine	A-2	9	1,090 ± 647	24.93 (23.75-28.17)	27,472 ± 19,725	6.6 ± 4.0	NC	1.99 ± 1.36
Phenylacetylglutamine	A-3	3	914 ± 439	25.47 (24.25-26.17)	19,989 ± 9,574	5.7 ± 3.6	NC	0.90 ± 0.25
Phenylacetylglutamine	C1	3	854 ± 51	24.31 (24.12-24.58)	20,726 ± 3,778	5.1 ± 1.0	NC	0.93 ± 0.06
Phenylacetylglutamine	C2	6	969 ± 496	24.59 (23.83-25.33)	28,068 ± 20,331	6.6 ± 4.5	NC	2.57 ± 2.82
Overall			997 ± 507	24.82 (23.75-28.17)	25,609 ± 16,771	6.3 ± 3.7		1.85 ± 1.78

Abbreviations: *n*, number; C_{max}, maximum plasma concentration; T_{max}, time of C_{max}; C_{last}, last quantifiable concentration; T_{last}, time of C_{last}; $T_{1/2}$, terminal half-life; $AUC_{[INF]}$, area-under the time curve from time zero to infinity.
*Data represents the mean ± SD; or individual values for $n < 2$.
†Data represents the mean variable value (range); or individual values for $n < 2$.

Four of 21 patients (19%) had detectable EBV DNA from PBMC at baseline. One patient with leiomyosarcoma and another patient with bladder cancer showed increase EBV viral DNA copy number after 5-AC treatment at dose level B (5-AC 75 mg/m^2; Supplemental Table S1). Three patients had reduced viral load after 5-AC treatment. Although exploratory, these mixed results do not allow us to draw any conclusions as to the usefulness of this assay in future studies.

Discussion

This report characterizes the safety and toxicity of 5-AC and phenylbutyrate together with pharmacokinetic analysis when 5-AC was combined with phenylbutyrate on different schedules and wide dose ranges in solid tumors. Generally, these different regimens were relatively well-tolerated and delivery of this range of dose regimens is feasible. No patients were taken off the study because of toxicity. The toxicity profile is consistent with the known adverse effects of 5-AC in hematologic malignancies (17, 18). Grade 3 toxicities were bone marrow suppression (likely 5-AC related) and neurocortical toxicity (likely phenylbutyrate related). Neutropenia occurred more often in dose level A1 when 5-AC was given at 25 mg/m^2/d for 2 weeks compared with dose level B when 5-AC was given at 75 mg/m^2/d for 1 week, the Food and Drug Administration–approved dose. The high rate of neutropenia (three of three, non-DLT neutropenia) in the first cohort of the study gave caution to the investigators, raising concerns that longer treatment with 5-AC would increase bone marrow suppression and recovery might be prolonged with the addition of an HDAC inhibitor. Longer treatment with 5-AC at lower doses caused neutropenia and anemia in some patients but did not delay subsequent cycles, although cycle length was lengthened to allow 3 weeks recovery after completing treatment before the next cycle began. The neurocortical toxicity

Table 5. Changes of tumor tissue methyltransferase activity after 5-AC

Patient ID	Tumor type	Dose level	Methyltransferase activity	
			Pretreatment	Posttreatment
1	Prostate	A1	0	0
3	Prostate	A1	442	448
4	Prostate	A-1	34	98
8	Liver	A-2	2,650	684
9	Kidney	A-2	20	0

Table 6. Changes of PBMC histone acetylation after treatments

Patient ID	Tumor type	Phenylbutyrate dose level, (mg/m^2/d)/D	Histone acetylation	
			H3	H4
11	Rectal	A-2 (400)/D6, D13	-	-
12	Renal	B1 (200)/D8 to D14	-	+
13	Colon		-	-
14	Prostate	A-3 (400)/D6, D13	-	-
15	Prostate		-	-
16	Breast		-	-
17	Mesothelia	B2 (400)/D8 to D14	-	-
19	Bladder		+	-
21	Renal		+	+
24	Colon	C1 (400)/D6, D13, D20	+	+
31	Rectal	C2 (400)/D6, D13, D20	+	-
32	Colon		+	+
33	Lung		+	+

NOTE: -, no changes; +, increase; D, days.

attributable to phenylbutyrate (3 of 27 patients) occurred at phenylbutyrate doses of 400 mg/kg/d on either the intermittent or continuous 7-day infusion schedules. This toxicity was consistent with previous reports (21).

Only one patient showed stable disease for 4.5 months and the MED was not defined in this study. Attempts to determine the MED through exploration of target inhibition was complicated by the need for tissue acquisition. At study initiation, over half of the patients consented to pretreatment and posttreatment biopsies. When accrual slowed while seeking patients agreeable to biopsies, the study moved ahead with accrual at the loss of tissue sample acquisition. However, evidence of biological effect/target inhibition was noted in some patients. Because of the availability of tissue pretreatment and posttreatment, only five samples were available for DNMT analysis and only half of the PBMC samples were analyzed. Although the primary end point to determine the safety and tolerability of this combination across a range of low 5-AC doses was met, the intensity of the trial in terms of the need for correlates hindered accrual. Many of the correlative studies were not conducted, preventing us from making any significant conclusions on target inhibition to determine the MED from a biological perspective.

The lack of clinical response by the combination of 5-AC and phenylbutyrate may be secondary to the following reasons: (*a*) solid tumors may have lower DNMT activity compared with myelodysplastic syndrome/acute myelogenous leukemia malignant cells. This is most likely the case for patients with prostate cancer, and might also reflect the lower S fraction of many patients with solid tumors. Nucleoside analogues require incorporation into DNA for activity. It is possible that 5-AC may inhibit DNMT only in tumors with high DNMT levels. Preclinical studies in breast cancer cell lines showed that gene re-expression occurred when DNMT was inhibited at levels of 90% or more (35). Unfortunately, we did not have epigenetic information from the patient with leiomyosarcoma who had stable disease after treatment. (*b*) Phenylbutyrate is a relatively weak HDAC inhibitor that only enhances mild to modest acetylation of histone in solid tumors. (*c*) Epigenetic therapies may also require longer exposure to these agents than cytotoxic therapies. In the late stage of cancer that patients enrolled on clinical trials typically represent, this requirement for longer exposure of the drugs, i.e., more cycles of treatments, for efficacy, is often not possible. Patients in this study had an average of four different chemotherapy regimens prior to this treatment and received only 1.7 cycles on average of this treatment in this study.

The administration of phenylbutyrate and 5-AC did not seem to alter the pharmacokinetics of either drug. 5-AC was eliminated rapidly from patients with a $t_{1/2}$ of 1.08 hours resulting in no accumulation after single daily dosing. The pharmacokinetics of 5-AC administered s.c. seemed to be linear with respect to C_{max} and $AUC_{[INF]}$ at the dose levels studied. These are consistent with our previous report (29) and in patients with hematologic malignancy (33). It was reported that only the higher doses of 5-AC (75 mg/m^2/d) could achieve plasma concentrations of 5 μmol/L that were shown to cause DNMT inhibition *in vitro* (29). Interestingly, in one patient with hepatocellular carcinoma, 5-AC at the 15 mg/m^2/d dose for 14 days suppressed DNMT activity by ~75%. Given the measurable inhibition of DNMT at lower concentrations than laboratory assays, which are typically with higher doses of shorter duration, this potentially suggests that pharmacodynamic effects could occur even at lower concentrations. This is consistent with recently published phase I results of 5-AC at a dose of 20 to 25 mg/m^2 daily for 10 days which achieved 4 to 8 months' duration of stable disease in 5 of 14 (35%) patients with advanced solid tumors (9). The disconnect between 5-AC plasma concentrations achieved at lower 5-AC doses and concentrations required *in vitro* to produce pharmacodynamic effects needs to be examined further. Several aspects for further study will involve characterizing the plasma protein binding of 5-AC and measurement of intracellular drug concentrations and in tumor.

The incidence of EBV infection in peripheral lymphocytes is ~29% in this small study. Two patients were found to have increased EBV viral load after 5-AC treatment at the dose level of 75 mg/m^2. One mechanism could be the demethylation of viral genome induced by 5-AC. Three patients showed reduced viral load. This is possibly secondary to the induced apoptosis or elimination of EBV-infected cells or B cells (34). Because of

the low incidence of EBV infection in solid tumor patient PBMC, EBV viral load may not be an ideal surrogate for DNMT inhibition.

We were not able to analyze all the tumor tissues or PBMCs for the correlative studies because of technical difficulties. From the available data, in one example, it seems that 5-AC could inhibit DNMT when the baseline enzyme activity is high. However, due to these considerations, this assay may not be optimal to confirm if demethylation occurred after 5-AC treatments. Tumor tissue or PBMC global gene DNA methylation analysis may be more reliable or direct to answer this specific question as how much epigenetic changes happened (36).

In the past several years, extensive and rapid drug development on HDAC inhibitors has lead to the approval of vorinostat (Zolinza; Merck, Inc.) for the treatment of refractory cutaneous T-cell lymphoma (37). More than 10 HDAC inhibitors are in phase I/II clinical trials. Many of them are orally administered (38, 39). Clinical interest in phenylbutyrate has waned given its cumbersome oral and intravenous administration, and based on this and other studies, we closed the study as there was little rationale to proceed with trials using phenylbutyrate in the treatment of solid malignancies. Despite not meeting our original goals for this study, we felt that we had explored a wide range of schedules/doses of the more clinically relevant agent, 5-AC.

In summary, the combinations of 5-AC and phenylbutyrate in three dose levels were generally well tolerated and safe, but no obvious clinical responses were achieved. The administration of phenylbutyrate and 5-AC did not seem to alter the pharmacokinetics of either drug. In contrast to clinical response, tumor tissue DNMT inhibition was achieved at a lower dose of 5-AC in one patient, suggesting that further studies at low doses of this drug might be warranted. PBMC histone acetylation induction was observed in some patients, but could not provide conclusive evidence for targeted response based on these limited correlative studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the following people from Johns Hopkins University for their support during both clinical trials: Rana Sullivan, Kathleen Burks, and Suzanne Dolan for nursing support; Bettye Carr and Jill Stewart for data management; Susan Davidson for quality assurance of the pharmacokinetic data contained in the manuscript; and Jatandra Birney, Carol Hartke, Ping He, Alex Mnatsakanyan, and Yelena Zabelina for their assistance in the pharmacokinetic quantitation.

References

1. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687–92.
2. Worm J, Guldberg P. DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy. J Oral Pathol Med 2002;31:443–9.
3. Glozak MA, Seto E. Histone deacetylases and cancer. Oncogene 2007;26:5420–32.
4. Razin A, Riggs AD. DNA methylation and gene function. Science 1980;210:604–10.
5. Rountree MR, Bachman KE, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. Oncogene 2001;20:3156–65.
6. Nan X, Ng HH, Johnson CA, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 1998;393:386–9.
7. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999;21:103–7.
8. Esteller M. DNA methylation and cancer therapy: new developments and expectations. Curr Opin Oncol 2005;17:55–60.
9. Braiteh F, Soriano AO, Garcia-Manero G, et al. Phase I study of epigenetic modulation with 5-azacytidine and valproic acid in patients with advanced cancers. Clin Cancer Res 2008;14:6296–301.
10. Creusot F, Acs G, Christman JK. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2′-deoxycytidine. J Biol Chem 1982;257:2041–8.
11. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J. Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. Anticancer Drugs 1997;8:358–68.
12. Pohlmann P, DiLeone LP, Cancella AI, et al. Phase II trial of cisplatin plus decitabine, a new DNA hypomethylating agent, in patients with advanced squamous cell carcinoma of the cervix. Am J Clin Oncol 2002;25:496–501.
13. Quagliana JM, O'Bryan RM, Baker L, et al. Phase II study of 5-azacytidine in solid tumors. Cancer Treat Rep 1977;61:51–4.
14. Srinivasan U, Reaman GH, Poplack DG, Glaubiger DL, LeVine AS. Phase II study of 5-azacytidine in sarcomas of bone. Am J Clin Oncol 1982;5:411–5.
15. Dover GJ, Charache S, Boyer SH, Vogelsang G, Moyer M. 5-Azacytidine increases HbF production and reduces anemia in sickle cell disease: dose-response analysis of subcutaneous and oral dosage regimens. Blood 1985;66:527–32.
16. Christman JK, Mendelsohn N, Herzog D, Schneiderman N. Effect of 5-azacytidine on differentiation and DNA methylation in human promyelocytic leukemia cells (HL-60). Cancer Res 1983;43:763–9.
17. Silverman LR, Holland JF, Weinberg RS, et al. Effects of treatment with 5-azacytidine on the *in vivo* and *in vitro* hematopoiesis in patients with myelodysplastic syndromes. Leukemia 1993;7 Suppl 1:21–9.
18. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 2002;20:2429–40.
19. Gore SD, Carducci MA. Modifying histones to tame cancer: clinical development of sodium phenylbutyrate and other histone deacetylase inhibitors. Expert Opin Investig Drugs 2000;9:2923–34.
20. Gilbert J, Baker SD, Bowling MK, et al. A phase I dose escalation and bioavailability study of oral sodium phenylbutyrate in patients with refractory solid tumor malignancies. Clin Cancer Res 2001;7:2292–300.
21. Carducci MA, Gilbert J, Bowling MK, et al. A phase I clinical and pharmacological evaluation of sodium phenylbutyrate on an 120-h infusion schedule. Clin Cancer Res 2001;7:3047–55.
22. DiGiuseppe JA, Weng LJ, Yu KH, et al. Phenylbutyrate-induced G1 arrest and apoptosis in myeloid leukemia cells: structure-function analysis. Leukemia 1999;13:1243–53.
23. McGrath-Morrow SA, Stahl JL. G(1) Phase growth arrest and induction of p21(Waf1/Cip1/Sdi1) in IB3-1 cells treated with 4-sodium phenylbutyrate. J Pharmacol Exp Ther 2000;294:941–7.
24. Robertson KD, Hayward SD, Ling PD, Samid D, Ambinder RF. Transcriptional activation of the Epstein-Barr virus latency C promoter after 5-azacytidine treatment: evidence that demethylation at a single CpG site is crucial. Mol Cell Biol 1995;15:6150–9.
25. Ben-Sasson SA, Klein G. Activation of the Epstein-Barr virus genome by 5-aza-cytidine in latently infected human lymphoid lines. Int J Cancer 1981;28:131–5.
26. Chan AT, Tao Q, Robertson KD, et al. Azacitidine induces demethylation of the Epstein-Barr virus genome in tumors. J Clin Oncol 2004;22:1373–81.
27. Robertson KD, Ambinder RF. Methylation of the Epstein-Barr virus genome in normal lymphocytes. Blood 1997;90:4480–4.
28. Shahabuddin M, Volsky B, Kim H, Sakai K, Volsky DJ. Regulated expression of human immunodeficiency virus type 1 in human glial cells: induction of dormant virus. Pathobiology 1992;60:195–205.
29. Rudek MA, Zhao M, He P, et al. Pharmacokinetics of 5-azacitidine administered with phenylbutyrate in patients with refractory solid tumors or hematologic malignancies. J Clin Oncol 2005;23:3906–11.
30. Zhao M, Rudek MA, He P, et al. Quantification of 5-azacytidine in plasma by electrospray tandem mass spectrometry coupled with high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2004;813:81–8.

31. Phuphanich S, Baker SD, Grossman SA, et al. Oral sodium phenylbutyrate in patients with recurrent malignant gliomas: a dose escalation and pharmacologic study. Neuro Oncol 2005;7: 177–82.

32. Rhee I, Jair KW, Yen RW, et al. CpG methylation is maintained in human cancer cells lacking DNMT1. Nature 2000;404:1003–7.

33. Gore SD, Baylin S, Sugar E, et al. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 2006;66:6361–9.

34. Yang J, Tao Q, Flinn IW, et al. Characterization of Epstein-Barr virus-infected B cells in patients with posttransplantation lymphoproliferative disease: disappearance after rituximab therapy does not predict clinical response. Blood 2000; 96:4055–63.

35. Ferguson AT, Vertino PM, Spitzner JR, Baylin SB, Muller MT, Davidson NE. Role of estrogen receptor gene demethylation and DNA methyltransferase.DNA adduct formation in 5-aza-2′ deoxycytidine-induced cytotoxicity in human breast cancer cells. J Biol Chem 1997;272: 32260–6.

36. Yegnasubramanian S, Haffner MC, Zhang Y, et al. DNA hypomethylation arises later in prostate cancer progression than CpG island hypermethylation and contributes to metastatic tumor heterogeneity. Cancer Res 2008;68: 8954–67.

37. Marchion D, Munster P. Development of histone deacetylase inhibitors for cancer treatment. Expert Rev Anticancer Ther 2007;7: 583–98.

38. Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 2007;5:981–9.

39. Carew JS, Giles FJ, Nawrocki ST. Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. Cancer Lett 2008;269:7–17.