RESEARCH ARTICLE

Combination Epigenetic Therapy Has Efficacy in Patients with Refractory Advanced Non–Small Cell Lung Cancer

Rosalyn A. Juergens[1], John Wrangle[1], Frank P. Vendetti[3], Sara C. Murphy[1], Ming Zhao[1], Barbara Coleman[1], Rosa Sebree[1], Kristen Rodgers[2], Craig M. Hooker[1], Noreli Franco[1], Beverly Lee[1], Salina Tsai[4], Igor Espinoza Delgado[5], Michelle A. Rudek[1], Steven A. Belinsky[6], James G. Herman[1], Stephen B. Baylin[1], Malcolm V. Brock[2], and Charles M. Rudin[1]
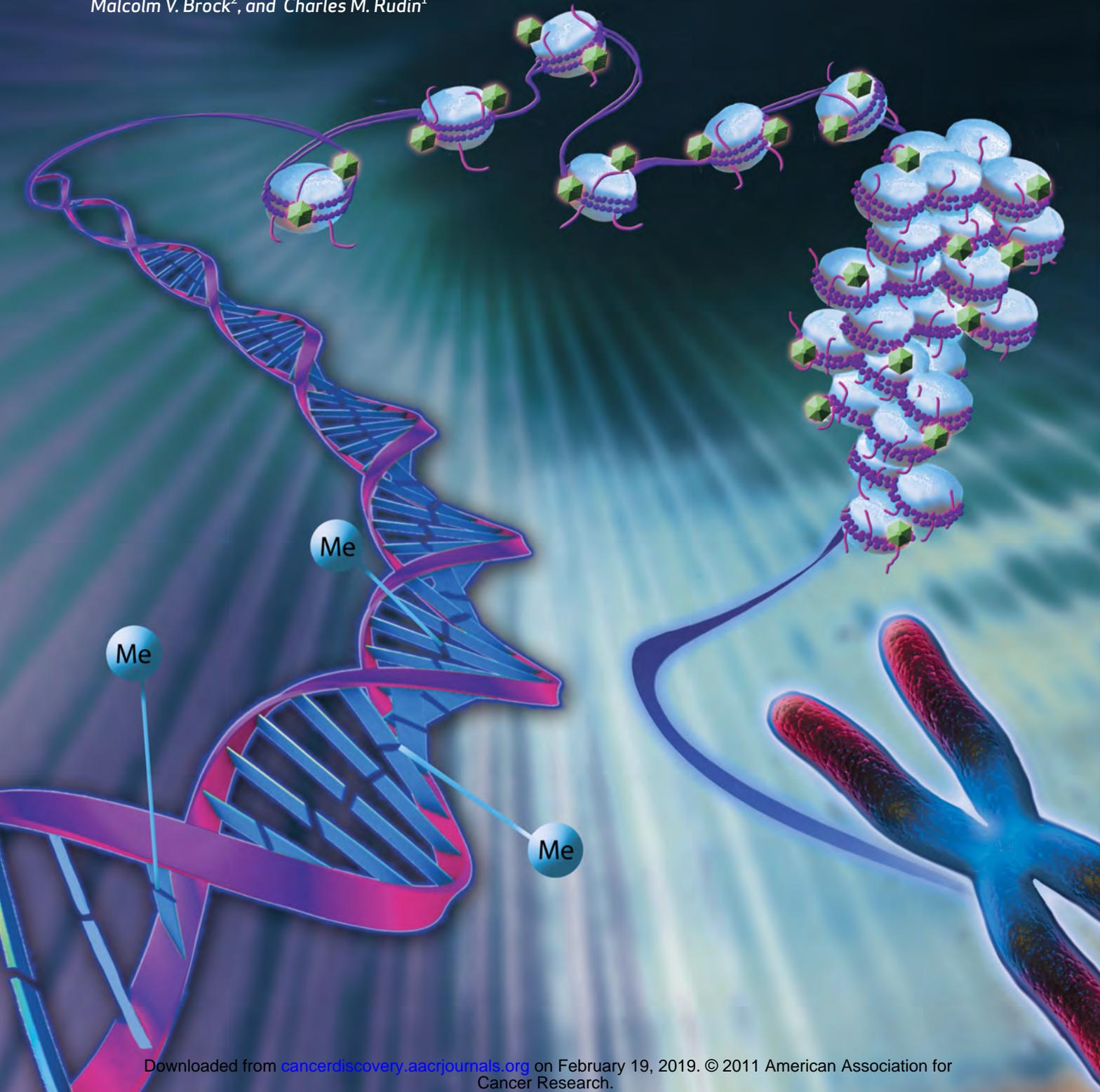
ABSTRACT   Epigenetic alterations are strongly associated with the development of cancer. We conducted a phase I/II trial of combined epigenetic therapy with azacitidine and entinostat, inhibitors of DNA methylation and histone deacetylation, respectively, in extensively pretreated patients with recurrent metastatic non–small cell lung cancer. This therapy is well tolerated, and objective responses were observed, including a complete response and a partial response in a patient who remains alive and without disease progression approximately 2 years after completing protocol therapy. Median survival in the entire cohort was 6.4 months (95% CI 3.8–9.2), comparing favorably with existing therapeutic options. Demethylation of a set of 4 epigenetically silenced genes known to be associated with lung cancer was detectable in serial blood samples in these patients and was associated with improved progression-free ($P = 0.034$) and overall survival ($P = 0.035$). Four of 19 patients had major objective responses to subsequent anticancer therapies given immediately after epigenetic therapy.

SIGNIFICANCE: This study demonstrates that combined epigenetic therapy with low-dose azacitidine and entinostat results in objective, durable responses in patients with solid tumors and defines a blood-based biomarker that correlates with clinical benefit. *Cancer Discovery; 1(7);* 598–607. ©*2011 AACR.*

INTRODUCTION

Aberrant epigenetic regulation of gene expression plays a fundamental role in oncogenesis and cancer progression (1). Recent data have strongly implicated epigenetic changes as a key determinant in the maintenance of subpopulations of cancer cells with high-level resistance to cytotoxic therapy and potent tumorigenic capacity (2). Multiple levels of epigenetic silencing have been defined, among which two of the most fundamental are DNA methylation and chromatin deacetylation (3). Unlike oncogenic mutations, which are fixed in the cancer genome, epigenetic alterations are potentially reversible, offering a unique therapeutic opportunity. Nonetheless, initial clinical evaluations of epigenetically targeted agents in patients with solid tumors have, in general, been disappointing.

Two cytidine analogs, azacitidine and decitabine, inhibit DNA methyltransferase activity upon incorporation into DNA, resulting in the loss of DNA methylation. Both of these agents are approved for use in patients with myelodysplastic syndrome. Initial studies of these agents in patients with solid tumors were associated with extensive toxicity and minimal efficacy when used at or near the maximally tolerated dose (4). However, the concentration required to reverse tumor-specific DNA methylation is much lower than that to produce maximal cytotoxicity. The cell-cycle arrest and cytotoxicity associated with high-level exposure to cytidine analogs may actually limit the reversal of DNA methylation state, which requires replicative incorporation into genomic DNA. For azacitidine and decitabine, both clinical efficacy and long-term tolerance improved in patients with myelodysplastic syndrome when doses well below maximally tolerated dose levels were used (5).

Multiple histone deacetylase (HDAC) inhibitors are also in clinical development, and two have now been approved in the treatment of another hematologic malignancy, cutaneous T-cell lymphoma (6). Single-agent activities of HDAC inhibitors in patients with solid tumor have been modest (7, 8). Current clinical development efforts are primarily focused on combination studies with cytotoxic agents on the basis in part of the ability of these agents to modify the acetylation state of a large number of nonhistone targets (9). Although the expression of many genes is influenced by HDAC inhibition alone, re-expression of densely hypermethylated, epigenetically silenced genes is not typically observed in the absence of a demethylating agent (10, 11). Preclinical models suggest that combinatorial epigenetic targeted therapy inhibiting both DNA methyltransferase and HDAC activity can synergistically reactivate gene expression and result in tumor response (10–13). In patients with myelodysplastic syndrome/leukemia treated with low-dose azacitidine and HDAC inhibitors, recent data have defined well-tolerated combination regimens and suggest promising activity (14, 15).

Entinostat is an orally bioavailable benzamidine HDAC inhibitor. In contrast to several other HDAC inhibitors in clinical development, entinostat has relatively focused inhibitory specificity for the class I HDACs 1 and 3 (16). Class I HDACs are found preferentially in the nucleus and regulate the acetylation state of DNA-associated histones. Other classes of HDACs shuttle between the nucleus and cytoplasm, or are predominantly cytoplasmic, and target a much broader array of protein targets (17, 18). Combination studies involving entinostat and a variety of anticancer agents are ongoing.

Authors' Affiliations: [1]Department of Oncology, [2]Department of Surgery, [3]Anti-Cancer Drug Development Graduate Training Program, and [4]Department of Radiology, Johns Hopkins University, Baltimore, Maryland; [5]Investigational Drug Branch, National Cancer Institute, Bethesda, Maryland; and [6]Lovelace Respiratory Research Institute, Albuquerque, New Mexico

Corresponding Authors: Charles M. Rudin, MD, PhD, Johns Hopkins University, Cancer Research Building 2, Room 544, 1550 Orleans Street, Baltimore, MD 21231. E-mail: rudin@jhmi.edu; or Malcolm V. Brock, MD, Johns Hopkins University, Cancer Research Building 1, Room 542, 1650 Orleans Street, Baltimore, MD 21231. E-mail: mbrock1@jhmi.edu

We recently evaluated the prognostic implications of promoter hypermethylation of a series of genes previously implicated as targets of tumor-specific epigenetic silencing in early stage non–small cell lung cancer (NSCLC) (19). Analysis of DNA methylation in tumors and mediastinal lymph nodes from a series of patients with surgically resected stage I NSCLC defined several prognostic markers associated with rapid tumor recurrence. Four gene targets of tumor-specific epigenetic silencing, *CDKN2a*, *CDH13*, *APC*, and *RASSF1a*, were identified as strongly associated with disease recurrence and death, both singly and in combination. Methylation of any 2 of these 4 target genes in tumor and mediastinal lymph nodes conferred a markedly worse prognosis in patients with stage I lung cancer ($P < 0.001$), similar to patients with stage III disease (19).

Together these observations suggest that the reversal of aberrant tumor-specific epigenetic silencing via the use of low-dose azacitidine and entinostat as defined in patients with myelodysplastic syndrome (15) might have clinical activity in advanced lung cancer and that this activity might correlate with effects on these defined epigenetically regulated target genes. We initiated a phase I/II study to test these hypotheses in patients with progressive, metastatic NSCLC.

RESULTS

Patient Characteristics

Ten patients participated in the phase I portion of this study; 3 patients received 30 mg/m^2/d azacitidine, and 7 received 40 mg/m^2/d azacitidine. Entinostat was administered to all patients at a fixed dose of 7 mg on days 3 and 10 of each cycle. In total, 42 patients, including the 7 patients from the phase I portion, were treated at the phase II azacitidine dose of 40 mg/m^2. This article summarizes data on all 45 phase I and II patients. Demographic characteristics are provided in Table 1.

The most common reason for study discontinuation of all patients receiving combination therapy was disease progression (39 of 45; 87%). The median number of treatment cycles was 2 (range, 1–18) and the median treatment duration was 52 days (range, 7–507 days).

Safety

Dose-limiting toxicities None of the 3 patients in the 30 mg/m^2 azacitidine cohort experienced dose-limiting toxicities (DLT). One patient in the 40 mg/m^2 azacitidine cohort withdrew from the study during the first week because of decreasing performance status and was replaced. None of the remaining 6 phase I patients had DLTs. In an effort to remain at an epigenetically targeted dose rather than a directly cytotoxic dose, the maximal dose planned in the phase I dose escalation was 40 mg/m^2. The recommended phase II dose was therefore defined as 40 mg/m^2 azacitidine given on days 1 to 6 and 8 to 10 plus 7 mg of entinostat given on days 3 and 10 of each 28-day cycle.

Adverse events All patients experienced at least one treatment-related adverse event. Toxicity attributions were assigned by the principal investigator. The most common treatment-related nonhematologic adverse events included low-grade skin/injection-site reactions (93%), fatigue (71%),

Table 1. Demographic characteristics

Characteristic	Patients (N = 45)
Age	
Median (range)	64 (46–80)
Sex	
Male/female	23/22
Race	
White/black/Asian	39/8/1
Smoking status	
Active	2
Former	38
Never	5
Histology	
Adenocarcinoma	34
Squamous	9
NSCLC not otherwise specified	2
Median number of previous therapies	3

nausea (73%), vomiting (40%), constipation (36%), anorexia (29%), electrolyte disturbances (29%), and hyperglycemia (22%). These adverse events are anticipated toxicities of either azacitidine or entinostat and generally required no intervention except the administration of antiemetics and other agents for gastrointestinal side effects, which were easily medically managed. Anemia was the most common hematologic toxicity (40%). Lymphopenia and thrombocytopenia were each observed in 27% of patients.

Grade 3 or 4 toxicities were seen in 28% of patients during cycle 1 (Table 2). The most common grade 3 or 4 toxicity was fatigue. Grade 3 or 4 hematologic toxicities were transient and generally asymptomatic. Four patients with anemia all improved after a one-time red blood cell transfusion. There were no correlations between the worst grade of toxicity and azacitidine or entinostat exposure ($P > 0.05$).

Pharmacokinetics

Pharmacokinetic data were obtained from 40 of the 42 patients treated at an azacitadine dose level of 40 mg/m^2/d. As previously reported, azacitidine was rapidly absorbed and eliminated with the time to maximal concentration (T_{max}) occurring at 0.50 hours (median; range, 0.25–2.00 hours) and half-life ($t_{1/2}$) of 1.12 ± 1.06 hours (average $\pm$ SD). The large variability in $t_{1/2}$ is in part attributable to patients who had detectable azacitidine concentrations at 6 (n = 11) or 8 ($n = 3$) hours. Maximum concentration (C_{max}) and area under the curve ($AUC_{0-\infty}$) for azacitidine were 468 ± 241 ng/mL and 675 ± 290 ng*h/mL, respectively. Entinostat concentrations in cycle 1 were 0.84 ± 0.23 ng/mL on day 10 (pretreatment) and 1.10 ± 0.34 ng/mL on day 17. Entinostat concentrations were detectable in 30% of patients before they began cycle 2 (day 29). In those patients, the residual concentrations were 0.66 ± 0.18 ng/mL.

Table 2. Grade 3 and 4 toxicities

Toxicities	Grade 3 Cycle 1 n = 45		Grade 3 Cycle ≥ 2 n = 32		Grade 4 Cycle 1 n = 45		Grade 4 Cycle ≥ 2 n = 32	
	n	%	n	%	n	%	n	%
Hematologic toxicities								
Anemia	3	6.7	1	3.1	—	—	—	—
Leukopenia	1	2.2	2	6.3	1	2.2	—	—
Lymphopenia	4	8.9	3	9.4	1	2.2	—	—
Neutropenia	—	—	3	9.4	1	2.2	—	—
Thrombocytopenia	1	2.2	—	—	—	—	—	—
Gastrointestinal symptoms								
Abdominal pain	1	2.2	—	—	—	—	—	—
Constipation	1	2.2	1	3.1	—	—	—	—
Diarrhea	1	2.2	—	—	—	—	—	—
Nausea	2	4.4	—	—	—	—	—	—
Vomiting	1	2.2	—	—	—	—	—	—
Electrolyte disturbances								
Hypokalemia	—	—	1	3.1	—	—	—	—
Hyponatremia	3	6.7	—	—	—	—	—	—
Generalized symptoms								
Anorexia	2	4.4	—	—	—	—	—	—
Dyspnea	1	2.2	—	—	—	—	—	—
Fatigue	2	4.4	4	12.5	—	—	—	—
Weakness	—	—	1	3.1	—	—	—	—

Efficacy

One patient had a complete response that lasted 14 months (Fig. 1). A second patient had a partial response that lasted 8 months (Fig. 2). Ten patients had stabilization of disease of at least 12 weeks. One of these patients had stable disease for 18 months and another for 14 months, both with prolonged symptomatic improvement. Twenty-two patients had progressive disease after 2 cycles of therapy. Eleven patients were considered nonevaluable for efficacy because they did not complete one cycle of therapy. Two of these patients withdrew consent because of difficulty with traveling on the basis of their schedule. Four of these patients came off study for decreased performance status. The remaining 5 patients experienced early disease progression, including one patient with unsuspected brain metastases diagnosed 10 days into treatment.

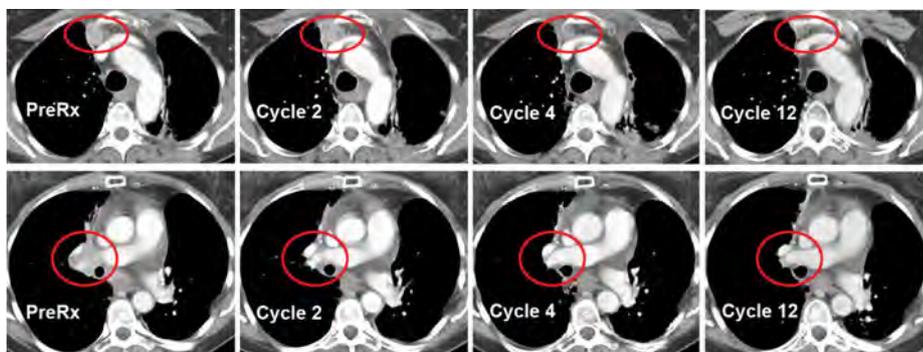The patient with the complete response was initially diagnosed with a stage IA adenocarcinoma but had rapid

Figure 1. Serial CT scan images from patient with a complete response. Scans show progressive resolution of a prevascular tumor (top) and a right hilar mass (bottom) during the course of 1 year on therapy. Red circles indicate areas of measurable disease.

Figure 2. Serial CT scan images from patient with a partial response. Scans demonstrate gradual resolution of sites of disease, including a lung mass, matted hilar lymph nodes, and one of the liver metastases. Although he was taken off study for treatment of an intercurrent small cell lung cancer, the patient remains alive and was without evidence of disease recurrence nearly 2 years after completion of this therapy. Red arrrows indicate areas of measurable disease.
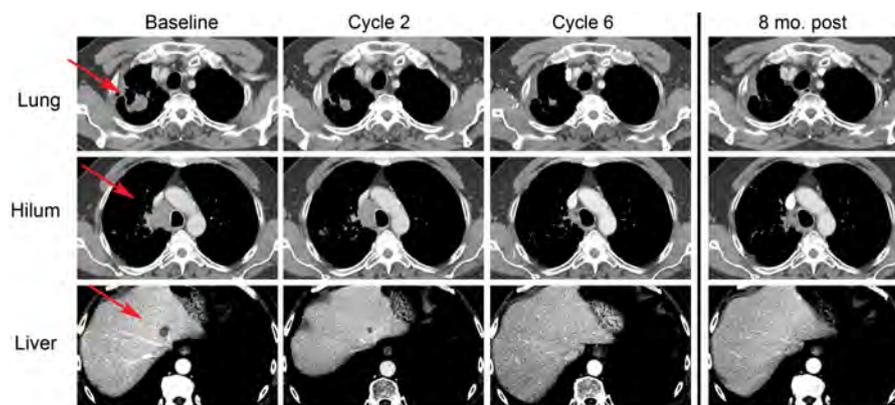
disease recurrence within 6 months of surgical resection. Analysis of the patient's original stage I tumor and mediastinal lymph nodes demonstrated a pattern of target gene hypermethylation previously defined as strongly predictive of early recurrence, with methylation of each of the 4 target genes detectable in either tumor, mediastinal lymph nodes, or both (19). In the 13 months between postsurgical recurrence and study enrollment, the patient experienced progressive disease during 3 different chemotherapy regimens. On combination epigenetic therapy, she experienced gradual improvement during a prolonged period of time and achieved a maximal response after approximately 6 months. After 14 months on treatment she was noted to have an enlarging solitary pulmonary nodule, which had been present as a 2-mm lesion at the time of study entry. The nodule was resected and appeared to represent a second primary NSCLC, molecularly distinct from her initial primary in that it harbored a *KRAS* mutation whereas her original tumor did not. Surgical exploration with biopsy at the time of resection revealed no viable tumor at previous sites of disease. She continued with no evidence of disease after the resection for nearly 12 months, followed by relapse and ultimately fatal progression of her *KRAS*-mutant tumor.

The patient with a partial response, also with 3 previous therapies for metastatic disease, had gradual complete radiographic resolution of multiple liver metastases and partial resolution

of lung and mediastinal disease during 8 cycles of therapy. He ultimately developed a new mediastinal nodule, which on biopsy proved to be a second primary tumor diagnosed as small cell lung cancer. This tumor was treated with etoposide, carboplatin, and concomitant radiation. The patient had no evidence of disease progression for 22 months after discontinuing epigenetic therapy and has been not been receiving any anticancer treatment for approximately 18 months, but has recently had localized disease recurrence of NSCLC in the chest. His liver metastases have completely resolved and remain undetectable more than 2 years after completion of therapy.

In an intent-to-treat analysis of all patients enrolled, we found that median progression-free survival was 7.4 weeks (95% CI, 7.0–8.0 weeks) and median overall survival was 6.4 months (95% CI, 3.8–9.2 months; Fig. 3). Median survival among patients who completed at least one cycle of epigenetic therapy was 8.6 months (95% CI, 5.5–12.2 months). The prolonged survival in patients with extensively pretreated metastatic NSCLC prompted an assessment of subsequent treatment. A total of 19 patients received at least one subsequent systemic treatment in the 6 months after they discontinued study (Fig. 4A), including 2 long-term survivors of 44 and 52 months postepigenetic therapy having received only one poststudy treatment regimen. Interestingly, 4 of the patients who received subsequent chemotherapy (21%) had major objective responses to the immediate subsequent therapy (Fig. 4B).
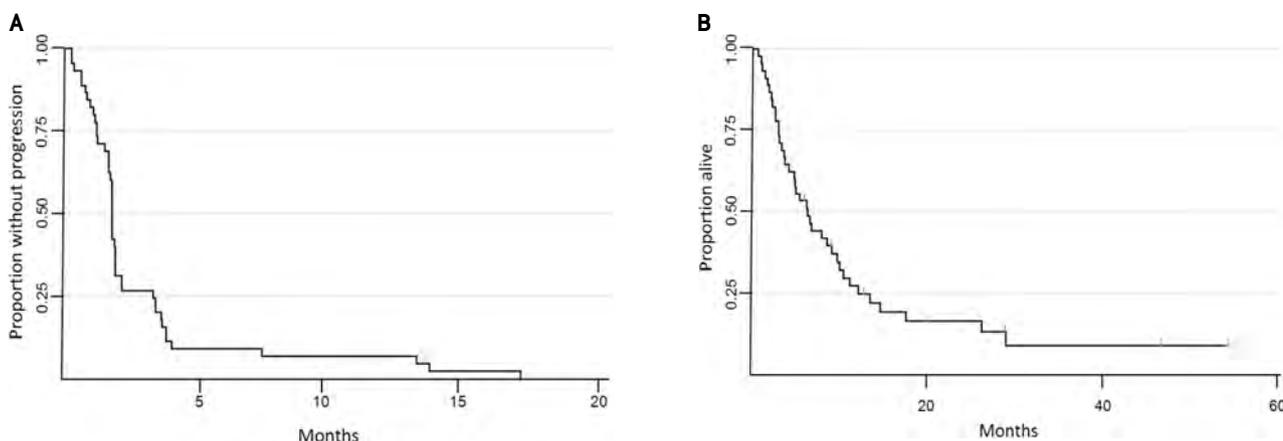

Figure 3. Kaplan–Meier survival analyses (intent-to-treat). All patients on study are included in these analyses. **A,** progression-free survival. **B,** overall survival.
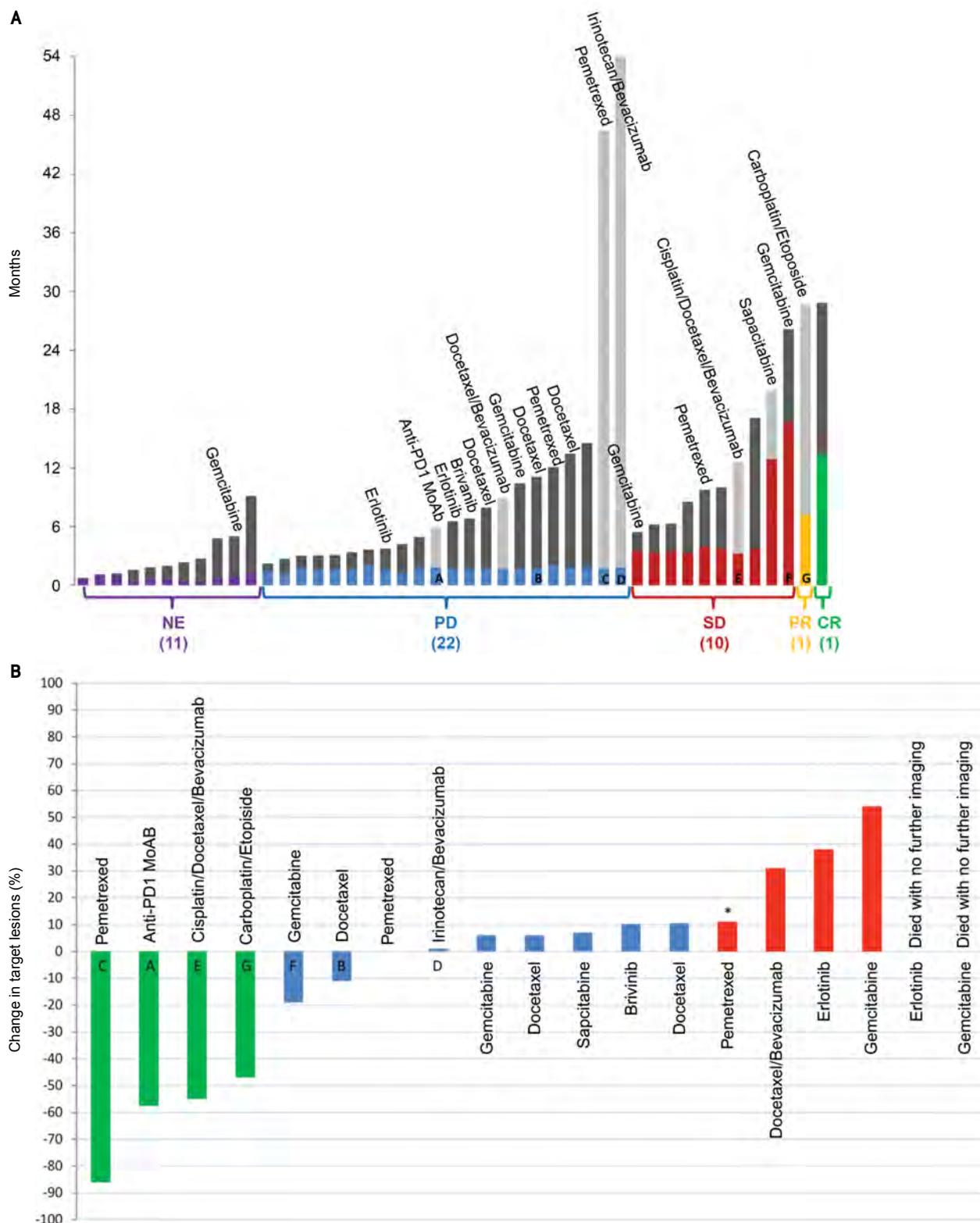
Figure 4. Survival, subsequent therapies, and response. **A,** duration of survival on and after protocol therapy. The height of the gray bar indicates duration of survival. Light gray bars indicate patients who are still alive; dark bars indicate patients who have died. The colored portion of the bar represents the duration of therapy received on trial. NE, nonevaluable for response; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response. If a patient received subsequent chemotherapy within 6 months, it is listed above the patient's survival bar. Letters identify corresponding patients in (**B**). **B,** waterfall plot of response to immediate subsequent therapy. The best change in defined target lesions to subsequent systemic anti-cancer treatment following epigenetic therapy is shown. Green, PR; blue, SD; red, PD. *Indicates progression defined by a new lesion. Two patients, indicated at right, died without follow-up imaging.

Biomarker Analyses

Our previous analysis of methylation status of tumor and mediastinal lymph nodes from patients with stage I NSCLC revealed 4 genes (*APC*, *RASSF1a*, *CDH13*, and *CDKN2a* as negative prognostic factors for survival (19). Promoter hypermethylation of at least 2 of these 4 genes in tumor and histologically normal lymph nodes was a strong predictor of early disease recurrence ($P < 0.001$). We hypothesized that the effect on methylation of these same 4 genes might define a population of lung cancer patients responsive to epigenetically targeted therapy. We did not have available pretreatment tumor biopsy material from patients on this study because pretreatment biopsies were not mandated, but we did have pretreatment and postcycle 1 plasma samples from study subjects.

Free plasma DNA concentration is known to be elevated in cancer patients and has proved to be a valuable source of tumor DNA for biomarker development, including detection of hypermethylated tumor suppressor genes in NSCLC patients (20–23). We therefore examined promoter methylation status in circulating DNA from patient plasma collected before therapy (day 0) and after 1 cycle of therapy (day 29). A total of 26 patients had sufficient circulating free DNA at both time points for analysis. Ten patients had at least 2 methylated target genes on day 0 and had a decrease in the level of methylation of 2 or more of these genes by day 29. Of these 10 "methylation signature"–positive patients, 8 (80%) had either stable disease or objective responses to epigenetic therapy. In contrast, of the 16 methylation signature-negative patients, only 4 (25%) had stable disease, and there were no objective responses. The median overall survival was 10.42 months for the methylation signature-positive cohort versus 6.54 months for the methylation signature-negative ($P = 0.035$; Fig. 5B). Progression-free survival was 3.32 months versus 1.71 for methylation signature-positive and negative cohorts, respectively ($P = 0.034$; Fig. 5A).

DISCUSSION

We report the first objective, durable responses in patients with solid tumors by using combined epigenetic therapy with a DNA methyltransferase inhibitor and an HDAC inhibitor. Objective responses to this therapy in this heavily pretreated population occurred in only 4% of the patients, but the antitumor responses that were observed were impressive. Several observations from this cohort of patients suggest that this therapy is quite distinct from previous experience with high-dose azacitidine and merits additional focused investigation.

Unlike participants in older clinical trials of azacitidine or decitabine in solid tumor patients (24–28), our patients received doses far below the maximally tolerated dose, permitting repetitive dosing over many months and avoiding the cytotoxicity associated with the high-dose regimens. Low-dose azacitidine or decitabine regimens have led to successful treatment of myelodysplastic syndrome (5, 14, 29, 30) with improved survival (31). Consistent with clinical trial observations in myelodysplastic syndrome (5), and in contrast to typical responses to cytotoxic chemotherapy, tumor responses in NSCLC patients improved gradually and progressively during several months of treatment. Intriguingly, the clinical responses produced were sustained even after cessation of epigenetic therapy. There was no evidence of relapse of the complete responder's original wild-type *KRAS* metastatic disease at the time of her death, 16 months after discontinuing epigenetic therapy. Similarly, at

present there has been no evidence of recurrence of the partial responder's hepatic metastases in the 2 years after epigenetic treatment was discontinued.

Another potential contributor to the responses seen in these patients is the methylation status of certain key genes in the tumor. Multiple studies have demonstrated the relevance of DNA methylation in lung cancer (32). The patient with a complete response demonstrated tumor-specific promoter hypermethylation in primary tumor and mediastinal lymph nodes in a pattern prognostic of poor survival in early stage lung cancer, which may define a subset of lung cancers driven by epigenetic mechanisms (19). The patient with partial response did not have baseline tumor available for analysis, but circulating DNA analysis from this patient confirms target gene methylation at baseline and demethylation with treatment. Both of these patients had methylation of 3 of 4 genes detectable in circulating DNA, with demethylation in all 3 with epigenetic therapy.

Analysis of free-circulating tumor DNA in the plasma of patients supports early demethylation as a potential predictor of clinical benefit from this therapy and is consistent with an on-target epigenetic mechanism of action. Evaluation of methylation changes in tumor DNA during cycle 1 of therapy as a predictor of clinical benefit should be included in future trials of epigenetically directed therapy. Other putative biomarkers of response to epigenetically targeted agents defined in recent studies could also be explored in this context (33).

Two of the patients described here developed molecularly and histologically distinct second lung cancers on therapy. This finding has not been observed in other patients in this study. Second primary cancers are common in patients with lung cancer and are associated with similar carcinogenic exposure throughout the lung field (34). Although it is difficult to entirely rule out the possibility of therapy-related adverse effects, secondary malignancies have not been reported in much larger series of patients receiving similar therapies for myelodysplastic syndrome (35, 36).

A limitation of the general applicability of this therapy is the need for subcutaneous injection of azacitidine on a daily basis. This was also a common cause of low-grade toxicities (e.g., injection-site reactions with localized erythema) on this study. The activity and bioavailability of an oral formulation of azacitidine has been recently reported in patients with hematologic malignancies (37). Other novel demethylating agents available in an oral formulation, including zebularine derivatives, have shown activity in experimental cancer models (38, 39). If bioequivalence can be demonstrated, these agents may have significant advantages over subcutaneous administration in terms of patient tolerance over prolonged courses of drug administration.

An important feature of this trial is combinatorial targeting of epigenetic silencing adding the HDAC inhibitor, entinostat. Preclinical data have demonstrated that although re-expression of epigenetically silenced target genes can be induced with inhibition of DNA methyltransferase alone, additional targeting of histone deacetylation results in more robust and persistent changes in gene expression (12, 13). In previous trials combining demethylating agents and HDAC inhibitors, investigators have used much less-potent HDAC inhibitors (40, 41). We hypothesize that these key features contributed to the responses observed.

The median survival of 6.4 months on azacitidine and entinostat in extensively pretreated NSCLC (median number of
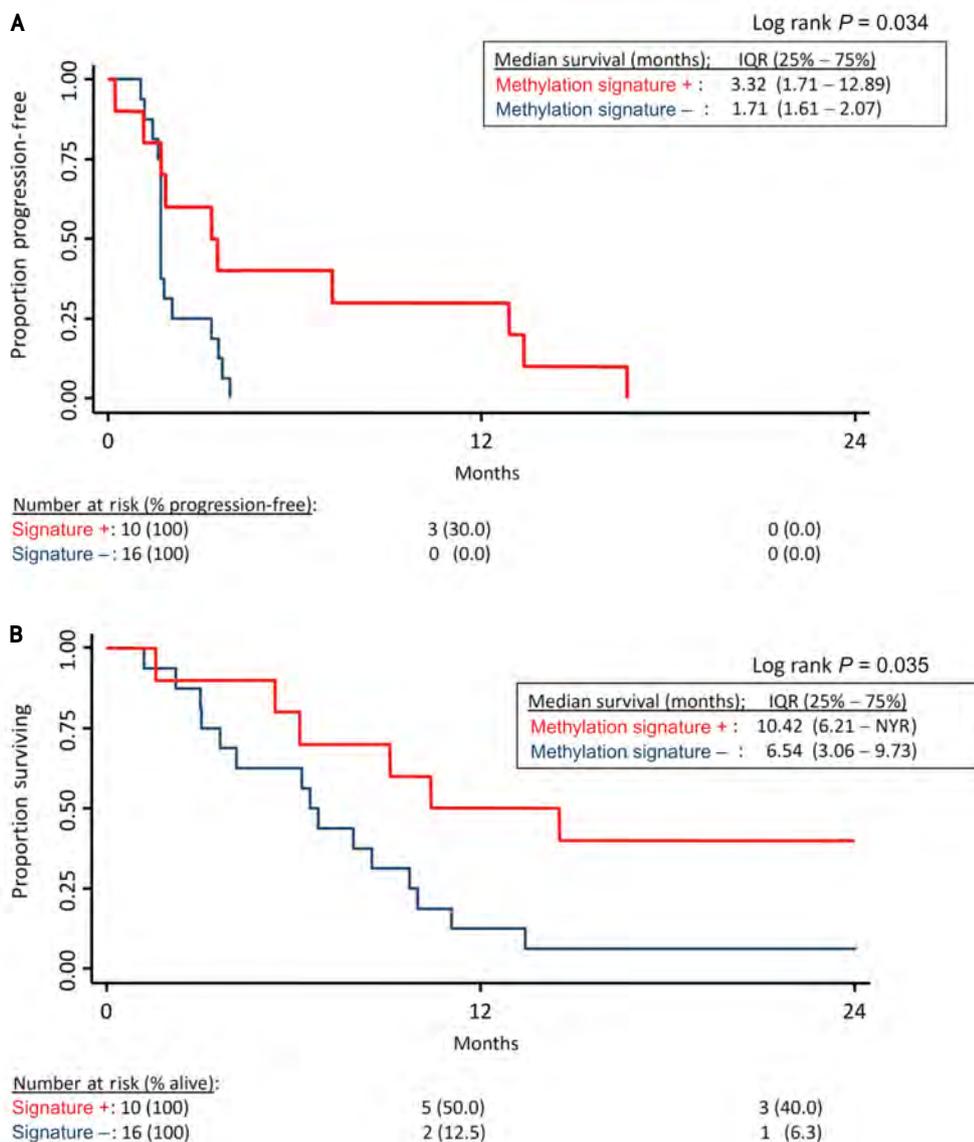
Figure 5. Survival stratified by target gene methylation status. Promoter methylation of *APC*, *CDH13*, *RASS1a*, and *CDKN2a* were evaluated in circulating plasma DNA from patients at pretreatment and on day 29. The red line indicates patients with pretreatment methylation of ≥2 of these 4 genes that demonstrate demethylation by day 29. The blue line indicates all other patients with detectable circulating DNA (total *n* = 26). **A,** overall survival. **B,** progression-free survival. IQR, interquartile range; NYR, not yet reached.

previous therapies, 3) is similar to the median survival previously noted for patients with 1 or 2 previous therapies treated with the only Food and Drug Administration-approved drug for this patient population, erlotinib (median survival 6.7 months, vs. 4.7 months for placebo control) (42). Stable changes in gene expression induced by epigenetically directed therapy could alter cancer cell sensitivity to subsequent cytotoxic therapy.

Recent data from the Settleman laboratory have defined drug-resistant "persisters" within clonal cancer cell populations highly sensitive to targeted therapy and that maintenance of these drug-resistant persisters is epigenetically regulated (2). Major objective responses to immediate subsequent therapies, even among patients with primary Response Evaluation Criteria in Solid Tumors (RECIST) progression on epigenetic therapy, have been observed and may have contributed to the exceptionally long survival among some patients on this study. Interestingly, the authors of a previous study also observed long-term survival in a patient after chemotherapy given after the DNA methyltransferase inhibitor decitabine (24, 43). These observations suggest a testable hypothesis that epigenetic therapy could prime cancers for response to subsequent cytotoxic therapy. Efforts to further refine characteristics of patients likely to benefit from this novel therapeutic approach, and to optimize this regimen to benefit additional cancer patients, are ongoing.

METHODS

Patient Population

This study enrolled adults with metastatic NSCLC with disease progression after at least one previous anticancer regimen for metastatic disease. Any number of previous therapies was allowed, and patients with treated brain metastases were included. Additional eligibility requirements included measurable disease per RECIST 1.0 (44), Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, life expectancy of ≥3 months, and adequate liver, renal, and bone marrow function. Patients were excluded if they had uncontrolled brain metastases or liver metastases replacing >30% of the liver parenchyma. Patients with HIV who were taking antiretroviral therapy also were excluded. This study was conducted according to the Declaration of Helsinki and with full Institutional Review Board approval. All participants provided written informed consent before participating. The study was registered with the National Institutes of Health (NCT00387465).

Study Design

The phase I component used a standard 3 + 3 patient cohort design to assess adverse events of the combination of azacitidine at 30 mg/m^2 or 40 mg/m^2 on days 1 to 6 and 8 to 10 with entinostat at a 7-mg fixed dose on days 3 and 10 of each 28-day cycle. On the basis of the strategy of exploring doses of azacitidine well below previously defined maximally tolerated doses, dose levels in phase I were prespecified as limited to no more than 40 mg/m^2 daily. The phase II component was a single-arm, 2-stage, open-label study designed to assess the response rate of the combination at the 40 mg/m^2/d dose of azacitidine.

Safety

Safety assessments included history and physical examinations, vital signs, ECOG performance status, adverse events, serum chemistry, and blood counts. Physical examinations were performed at screening every other week during cycle 1, monthly during cycle 2 and subsequent cycles, and at the final study visit. Laboratory analyses were performed at screening, every other week while the patient was receiving treatment, and at the final study visit. The severity of adverse events was graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events, version 3.0 (45). Adverse events judged possibly or probably related to azacitidine and entinostat administration were considered DLTs if they satisfied any of the following criteria: nonhematologic grade 3/4 toxicity except grade 3 nausea or vomiting only if unresponsive to therapy; grade 4 neutropenia, leukopenia, or lymphopenia; and decreased hemoglobin. A delay in treatment of greater than 2 weeks was also considered a DLT. The phase II dose was defined as the dose at which ≤30% of patients experienced DLTs during cycle 1 up to a prespecified maximal dose of 40 mg/m^2 of azacitidine.

Pharmacokinetics

For azacitidine, blood samples were obtained before treatment and at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hours after the first dose of azacitidine. Plasma samples were stored and processed as previously described with the use of liquid chromatography/tandem mass spectrometry (46, 47). Pharmacokinetic parameters were determined as previously described (46).

For entinostat, blood samples were collected on cycle 1 day 1 and then approximately 7 days after entinostat administration on days 10 and 17. Plasma concentrations of entinostat were measured with the use of liquid chromatography/tandem mass spectrometry as previously described (48). Samples were considered trough concentrations (C$_{min}$) if they were collected pretreatment concentrations on days 10 and 17.

Pharmacodynamic Correlates

Free-circulating plasma tumor DNA was evaluated for DNA methylation in each patient. Samples were collected before treatment and on days 10 and 29 of the study. Blood samples for methylation analysis were collected into citrate Vacutainer cell preparation tubes (BD).

DNA methylation, for all lung cancer, lymph node, and plasma DNA samples, was determined by methylation-specific PCR (MSP) and performed as previously described (49). Plasma samples were also analyzed by quantitative MSP with the use of Applied Biosystems StepOne qPCR machines, and qPCR or molecular beacon primer or primer and probe sets. CDH13 and RASSF1a methylation were analyzed with the use of MSP primers for quantitative analysis on the AB platform with 60°C as the annealing temperature. Molecular beacon assays were used to analyze APC and CDKN2a methylation, with β-actin for gene normalization, all with the use of 57°C as the annealing temperature.

All analyses were performed by investigators blinded to the origin of the samples. All reactions were performed in triplicate. $Ct_{genemax}$ is equal to the earliest cycle threshold for any sample in the dataset and $Ct_{gene1, 2, and 3}$ are used to denote samples run in triplicate. Relative methylation values were generated using the following equation:

$$\frac{[1/(Ct_{gene1} - Ct_{genemax}) + 1/(Ct_{gene2} - Ct_{genemax}) + 1/(Ct_{gene3} - Ct_{genemax})]}{[1/(Ct_{actin1} - Ct_{actinmax}) + 1/(Ct_{actin2} - Ct_{actinmax}) + 1/(Ct_{actin3} - Ct_{actinmax})]}$$

Efficacy

Efficacy variables, including objective response rate, progression-free survival, and overall survival, were evaluated for all patients treated at the phase II dose. Tumor response was assessed by the use of RECIST 1.0 after every 2 cycles of therapy (44). Response assessment to subsequent therapy was similarly determined on the basis of RECIST criteria and was performed by a single radiologist blinded to clinical data.

Statistical Analysis

Simple descriptive statistics were used to display the data on toxicity seen from azacitidine and entinostat in this patient population. Pharmacokinetic parameters were summarized with the use of descriptive statistics. The distributions of progression-free and overall survival were estimated with the Kaplan–Meier method (50). A Kruskal–Wallis test was used to determine the association between azacitidine and entinostat exposure and response and worst grade of toxicity. The method of Tukey–Kramer was used to adjust for multiple comparisons of mean values. The association between categorical variables was assessed using Fisher's exact test. All tests of hypotheses were conducted at 2-sided α = 0.05 level.

Disclosure of Potential Conflicts of Interest

There are no direct financial conflicts of interests for the prospective conduct of the clinical trial reported. R.A. Juergens and C.M. Rudin have consulted for Syndax. J.G. Herman and S.B. Baylin have consulted for MDxHealth. J.G. Herman, S.B. Baylin, and M.V. Brock have research support from MDxHealth, and hold a patent licensed to MDxHealth. These arrangements are managed by the Johns Hopkins University in accordance with its conflict of interest policies.

REFERENCES

1. Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–92.
2. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Cell 2010;141:69–80.
3. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
4. Glover AB, Leyland-Jones BR, Chun HG, Davies B, Hoth DF. Azacitidine: 10 years later. Cancer Treat Rep 1987;71:737–46.
5. Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, 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.
6. Howman RA, Prince HM. New drug therapies in peripheral T-cell lymphoma. Expert Rev Anticancer Ther 2011;11:457–72.
7. Vansteenkiste J, Van Cutsem E, Dumez H, Chen C, Ricker JL, Randolph SS, et al. Early phase II trial of oral vorinostat in relapsed or refractory breast, colorectal, or non–small cell lung cancer. Invest New Drugs 2008;26:483–8.
8. Schrump DS, Fischette MR, Nguyen DM, Zhao M, Li X, Kunst TF, et al. Clinical and molecular responses in lung cancer patients receiving Romidepsin. Clin Cancer Res 2008;14:188–98.

9. Marks PA. Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. Biochim Biophys Acta 2010;1799:717–25.

10. Shoemaker AR, Mitten MJ, Adickes J, Ackler S, Refici M, Ferguson D, et al. Activity of the Bcl-2 family inhibitor ABT-263 in a panel of small cell lung cancer xenograft models. Clin Cancer Res 2008;14:3268–77.

11. Jain HV, Meyer-Hermann M. The molecular basis of synergism between carboplatin and ABT-737 therapy targeting ovarian carcinomas. Cancer Res 2011;71:705–15.

12. Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, et al. Inhibition of DNA methylation and histone deacetylation prevents murine lung cancer. Cancer Res 2003;63:7089–93.

13. 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.

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

15. Fandy TE, Herman JG, Kerns P, Jiemjit A, Sugar EA, Choi SH, et al. Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies. Blood 2009;114:2764–73.

16. Hu E, Dul E, Sung CM, Chen Z, Kirkpatrick R, Zhang GF, et al. Identification of novel isoform-selective inhibitors within class I histone deacetylases. J Pharmacol Exp Ther 2003;307:720–8.

17. Johnstone RW, Licht JD. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? Cancer Cell 2003;4:13–8.

18. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006;5:769–84.

19. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glöckner S, et al. DNA methylation markers and early recurrence in stage I lung cancer. N Engl J Med 2008;358:1118–28.

20. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non–small cell lung cancer patients. Cancer Res 1999;59:67–70.

21. Sozzi G, Conte D, Leon M, Ciricione R, Roz L, Ratcliffe C, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. J Clin Oncol 2003;21:3902–8.

22. Sirera R, Bremnes RM, Cabrera A, Jantus-Lewintre E, Sanmartín E, Blasco A, et al. Circulating DNA is a useful prognostic factor in patients with advanced non–small cell lung cancer. J Thorac Oncol 2011;6:286–90.

23. Lee YJ, Yoon KA, Han JY, Kim HT, Yun T, Lee GK, et al. Circulating cell-free DNA in plasma of never smokers with advanced lung adenocarcinoma receiving gefitinib or standard chemotherapy as first-line therapy. Clin Cancer Res 2011;17:5179–87.

24. 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.

25. Vogler WR, Arkun S, Velez-Garcia E. Phase I study of twice weekly 5-azacytidine (NSC-102816). Cancer Chemother Rep 1974;58:895–9.

26. Lomen PL, Baker LH, Neil GL, Samson MK. Phase I study of 5-azacytidine (NSC-102816) using 24-hour continuous infusion for 5 days. Cancer Chemother Rep 1975;59:1123–6.

27. Weiss AJ, Stambaugh JE, Mastrangelo MJ, Laucius JF, Bellet RE. Phase I study of 5-azacytidine (NSC-102816). Cancer Chemother Rep 1972;56:413–9.

28. Quagliana JM, O'Bryan RM, Baker L, Gottlieb J, Morrison FS, Eyre HJ, et al. Phase II study of 5-azacytidine in solid tumors. Cancer Treat Rep 1977;61:51–4.

29. List AF. Treatment strategies to optimize clinical benefit in the patient with myelodysplastic syndromes. Cancer Control 2008;15(Suppl):29–39.

30. Lubbert M, Bertz H, Ruter B, Marks R, Claus R, Wäsch R, et al. Non-intensive treatment with low-dose 5-aza-2′-deoxycytidine (DAC) prior to allogeneic blood SCT of older MDS/AML patients. Bone Marrow Transplant 2009;44:585–8.

31. Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, Schoch R, et al. Efficacy of azacitidine compared with

that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol 2009;10:223–32.

32. Anglim PP, Alonzo TA, Laird-Offringa IA. DNA methylation-based biomarkers for early detection of non–small cell lung cancer: an update. Mol Cancer 2008;7:81.

33. Simo-Riudalbas L, Melo SA, Esteller M. DNMT3B gene amplification predicts resistance to DNA demethylating drugs. Genes Chromosomes Cancer; 50:527–34.

34. Brock MV, Alberg AJ, Hooker CM, Kammer AL, Xu L, Roig CM, et al. Risk of subsequent primary neoplasms developing in lung cancer patients with prior malignancies. J Thorac Cardiovasc Surg 2004;127:1119–25.

35. Gandhi L, Camidge DR, Ribeiro de Oliveira M, Bonomi P, Gandara D, Khaira D, et al. Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. J Clin Oncol 2011;29:909–16.

36. Rudin CM, Mauer A, Smakal M, Juergens R, Spelda S, Wertheim M, et al. Phase I/II study of pemetrexed with or without ABT-751 in advanced or metastatic non–small-cell lung cancer. J Clin Oncol 2011;29:1075–82.

37. Garcia-Manero G, Gore SD, Cogle C, Ward R, Shi T, Macbeth KJ, et al. Phase I study of oral azacitidine in myelodysplastic syndromes, chronic myelomonocytic leukemia, and acute myeloid leukemia. J Clin Oncol; 29:2521–7.

38. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 2003;95:399–409.

39. Herranz M, Martin-Caballero J, Fraga MF, Ruiz-Cabello J, Flores JM, Desco M, et al. The novel DNA methylation inhibitor zebularine is effective against the development of murine T-cell lymphoma. Blood 2006;107:1174–7.

40. Braiteh F, Soriano AO, Garcia-Manero G, Hong D, Johnson MM, Silva Lde P, 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.

41. Lin J, Gilbert J, Rudek MA, Zwiebel JA, Gore S, Jiemjit A, et al. A phase I dose-finding study of 5-azacytidine in combination with sodium phenylbutyrate in patients with refractory solid tumors. Clin Cancer Res 2009;15:6241–9.

42. Shepherd FA, Pereira J, Ciuleanu TE, Tan EH, Hirsh V, Thongprasert S, et al. A randomized placebo-controlled trial of erlotinib in patients with advanced non–small cell lung cancer following failure of 1st line or 2nd line chemotherapy. A National Cancer Institute of Canada Clinical Trials Group trial. Proc Am Soc Clin Oncol 2004;22:622s.

43. Momparler RL, Ayoub J. Potential of 5-aza-2′-deoxycytidine (Decitabine) a potent inhibitor of DNA methylation for therapy of advanced non–small cell lung cancer. Lung Cancer 2001;34 Suppl 4: S111–5.

44. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.

45. Trotti A, Colevas AD, Setser A, Rusch V, Jaques D, Budach V, et al. CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. Semin Radiat Oncol 2003;13:176–81.

46. Rudek MA, Zhao M, He P, Hartke C, Gilbert J, Gore SD, 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.

47. Zhao M, Rudek MA, He P, Hartke C, Gore S, Carducci MA, et al. Quantification of 5-azacitidine in plasma by electrospray tandem mass spectrometry coupled with high-performance liquid chromatography. J Chromatogr 2004;813:81–8.

48. Zhao M, Rudek MA, Mnasakanyan A, Hartke C, Pili R, Baker SD. A liquid chromatography/tandem mass spectrometry assay to quantitate MS-275 in human plasma. J Pharm Biomed Anal 2007;43:784–7.

49. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.

50. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–81.

CANCER DISCOVERY

AACR American Association for Cancer Research

Combination Epigenetic Therapy Has Efficacy in Patients with Refractory Advanced Non–Small Cell Lung Cancer

Rosalyn A. Juergens, John Wrangle, Frank P. Vendetti, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/2159-8290.CD-11-0214
Supplementary Material	Access the most recent supplemental material at: http://cancerdiscovery.aacrjournals.org/content/suppl/2011/11/15/2159-8290.CD-11-0214.DC1

Cited articles	This article cites 48 articles, 18 of which you can access for free at: http://cancerdiscovery.aacrjournals.org/content/1/7/598.full#ref-list-1
Citing articles	This article has been cited by 36 HighWire-hosted articles. Access the articles at: http://cancerdiscovery.aacrjournals.org/content/1/7/598.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/1/7/598. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.