Decitabine Effect on Tumor Global DNA Methylation and Other Parameters in a Phase I Trial in Refractory Solid Tumors and Lymphomas

David J. Stewart, Jean-Pierre Issa, Razelle Kurzrock, Maria I. Nunez, Jaroslav Jelinek, David Hong, Yasuhiro Oki, Zhong Guo, Sanjay Gupta, and Ignacio I. Wistuba

Abstract

Purpose: By hypomethylating genes, decitabine may up-regulate factors required for chemotherapeutic cytotoxicity. Platinum-resistant cells may have reduced expression of the copper/platinum transporter CTR1.

Experimental Design: Thirty-one patients with refractory malignancies received decitabine 2.5 to 10 mg/m^2 on days 1 to 5, and 8 to 12 or 15 to 20 mg/m^2 on days 1 to 5. Tumor was assessed for DNA methylation (by LINE assays), apoptosis, necrosis, mitoses, Ki67, DNA methyltransferase (DNMT1), CTR1, and p16.

Results: Febrile neutropenia was dose limiting. One thymoma patient responded. Decitabine decreased tumor DNA methylation (from median 51.2% predecitabine to 43.7% postdecitabine; $P = 0.01$, with effects at all doses) and in peripheral blood mononuclear cells (from 65.3-56.0%). There was no correlation between tumor and peripheral blood mononuclear cells. Patients starting decitabine ≤3 versus >3 months after last prior cytotoxic or targeted therapy had lower predecitabine tumor CTR1 scores ($P = 0.02$), higher p16 ($P = 0.04$), and trends ($P = 0.07$) toward higher tumor methylation and apoptosis. Decitabine decreased tumor DNMT1 for scores initially >0 ($P = 0.04$). Decitabine increased tumor apoptosis ($P < 0.05$), mitoses (if initially low, $P = 0.02$), and CTR1 (if initially low, $P = 0.025$, or if ≤3 months from last prior therapy, $P = 0.04$). Tumor CTR1 scores correlated inversely with methylation (r = −0.41, $P = 0.005$), but *CTR1* promoter was not hypermethylated. Only three patients had tumor *p16* promoter hypermethylation. P16 scores did not increase. Higher blood pressure correlated with lower tumor necrosis ($P = 0.03$) and a trend toward greater DNA demethylation ($P = 0.10$).

Conclusions: Exposure to various cytotoxic and targeted agents might generate broad pleiotropic resistance by reducing CTR1 and other transporters. Decitabine decreases DNA methylation and augments CTR1 expression through methylation-independent mechanisms.

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Hypermethylation by DNA methyltransferase (DNMT) helps regulate gene expression, and tumor suppressor gene hypermethylation promotes tumorigenesis (1–4). Altered gene methylation may also cause chemotherapy resistance (5). Many factors underlie chemotherapy resistance (6), but dose-response curve flattening at higher doses (7) suggests that deficiency of factors required for cytotoxicity may be particularly important. For example, platinum-resistant cells may have hypermethylation of the MLH1 mismatch repair gene that is important in triggering platinum cytotoxicity (8) or may have a pleiotropic reduction in transporters (9, 10) that is potentially reversible by the DNMT inhibitor decitabine (9). The copper transporter CTR1 contributes to cellular platinum uptake (11). Platinum exposure rapidly decreases CTR1 expression, thereby reducing further platinum influx (12).

DNMT inhibitors tested clinically include decitabine (5-aza-2′-deoxycytidine; refs. 2, 4, 13–16), 5-azacytidine (4), and MG-98 (17). Decitabine inhibits DNMT, depletes DNMT1 through proteosomal degradation (18), induces global DNA hypomethylation, and increases expression of specific genes through mechanisms both dependent on (2, 16) as well as independent of (2, 4) promoter hypomethylation.

Of administration schedules tested in leukemias, 1-hour low-dose decitabine infusions days 1 to 5 ± days 8 to 12 every 4 weeks may be particularly effective therapeutically (13–15, 19) and in demethylating DNA (14, 19). Low decitabine doses

Authors' Affiliation: M. D. Anderson Cancer Center, Houston, Texas
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Requests for reprints: David J. Stewart, Department of Thoracic/Head and Neck Medical Oncology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. Phone: 713-792-6363; Fax: 713-792-1220; E-mail: dstewart@mdanderson.org.
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Translational Relevance

(*a*) Decitabine reduces DNA methylation in solid tumors, and should be assessed for its ability to increase expression of factors required for efficacy of other agents. (*b*) Peripheral blood mononuclear cells should not be used as surrogates for decitabine effect in tumor. (*c*) Exposure within the previous 3 months to a wide range of chemotherapy and targeted agents is associated with decreased copper/platinum uptake transporter CTR1 and with a trend to increased DNA methylation. Hence, many agents might reduce subsequent platinum uptake. We will explore the possibility that other transporters are also down-regulated, that this is a mechanism underlying epigenetic broad cross-resistance, and that agents that do not require uptake into cells (e.g., antibodies) could combine more effectively with chemotherapy agents than do small molecules (which require uptake into cells). (*d*) Decitabine increases CTR1 expression, suggesting that it should be further evaluated for its ability to reduce platinum resistance.

(sufficient to cause DNA hypomethylation) seemed to be more effective than higher cytotoxic doses in leukemia and myelodysplasia (13–15). Low-dose decitabine can also restore hemoglobin F production in sickle cell anemia (4, 20).

Because low-dose daily decitabine is effective in leukemias, we defined the maximum tolerated dose of this schedule in patients with refractory solid tumors and lymphomas and assessed decitabine effect on tumor and peripheral blood mononuclear cell (PBMC) DNA methylation and on tumor necrosis, apoptosis, mitoses, Ki67, CTR1, DNMT1, and the tumor suppressor gene p16 (which may be inactivated by hypermethylation; ref. 21). Because drug delivery may vary with tissue blood flow (22) and because tumor blood flow is more sensitive to blood pressure than is normal tissue blood flow (23, 24), we also assessed effect on decitabine effect of day 1 systolic blood pressure (SBP). Drug uptake into tumors may also vary with tumor pH (25). Hence, we also assessed effect of factors that might be related to tumor pH, including serum lactate dehydrogenase (LDH; because LDH5 converts pyruvate to lactate in tumors; ref. 26), glucose (because administration of a glucose load may reduce tumor extracellular pH; ref. 27), CO_2 (because administration of a bicarbonate load may raise tumor pH; ref. 28), and chloride.

Materials and Methods

Eligibility criteria for this Institutional Review Board–approved protocol included written informed consent, solid tumor or lymphoma refractory to standard therapy, biopsiable tumor, and adequate organ function. Dose-limiting toxicity was defined as febrile neutropenia, grade 4 thrombocytopenia lasting >2 wk, treatment-related bleeding, or clinically significant ≥grade 3 nonhematologic toxicity occurring with the first therapy cycle. Decitabine was supplied by the National Cancer Institute Division of Cancer Treatment and Diagnosis under a Collaborative and Research Development Agreement. Patients received decitabine i.v. over 1 h daily, with ≥6 patients per cohort. Cohorts 1, 2, and 3 received decitabine 2.5, 5, and 10 mg/m^2/d days 1 to 5 and 8 to 12 of each 4-wk cycle. Because substantial myelosuppression (but less than maximum tolerated dose) was seen in cohort 3, and because updated data from leukemia studies (19) suggested that administration of low-dose decitabine days 1 to 5 was as effective as days 1 to 5 and 8 to 12, cohorts 4 and 5, respectively, received 15 and 20 mg/m^2/d on days 1 to 5 only. Granulocyte colony-stimulating factor was added for cohort 5. Tumor biopsies were done on all patients prior to decitabine and again on cycle 1 day 12 (within a few hours of the final cycle 1 dose for cohorts 1 to 3 and 7 d after last decitabine for cohorts 4 and 5). PBMCs were collected on the days of tumor biopsies. Computed tomography scans to evaluate tumor size were first repeated after cycle 1.

Tumors were characterized histopathologically with respect to tumor type, % necrosis, number of mitoses, % of cells with nuclear staining for Ki67 (29), and apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (30). Global tumor and PBMC DNA methylation (% of CpG islands methylated) was assessed by LINE assays (31). Change in DNA methylation was calculated by dividing absolute change (day 12 minus day 1) by the day 1 value and multiplying by 100. Promoter methylation for *p16* and *CTR1* genes was assessed by pyrosequencing, as previously described (32).

Table 1. Patient characteristics

Patient characteristic	No. of patients
Total	31
Gender: male	17
Female	14
Median age (range)	53 (20-75)
Tumor type: malignant melanoma	6
Renal cell carcinoma	3
Breast carcinoma	4
Cutaneous T-cell lymphoma/ mycosis fungoides	3
Thymoma/thymic carcinoma	4
Adenocystic carcinoma	2
Head & neck squamous carcinoma	2
Neuroendocrine carcinomas	2
Desmoplastic tumor	1
Other carcinomas	4
Dose Level (mg/m^2/d): 2.5 × 10 d	6
5 × 10 d	6
10 × 10 d	7
15 × 5 d	6
20 × 5 days	6
Months from last therapy: median (range)	
Last cytotoxic therapy	3 (1-31)
Last platinum (*n* = 22)	9.5 (1.5-50)
Last cytotoxic or targeted therapy	2 (1-18)
No. of prior systemic regimens: median (range)	5 (1-14)
No. of prior targeted agents: median (range)	2 (0-6)
No. of patients previously treated with targeted agents:	
Thalidomide	5
Bevacizumab	7
Interferon α	9
EGFR inhibitor (gefitinib, erlotinib, cetuximab, PKI-166)	8
Histone deacetylase inhibitors	3
No. of other prior targeted agents	21

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Table 2. Grade ≥3 toxicity with first cycle decitabine

Decitabine mg/m²/d × no. days	2.5 × 10	5 × 10	10 × 10	15 × 5	20 × 5
No. of cycles	6	6	7	6	6
Toxicity:	No. of cycles with toxicity				
Neutropenia: grade 3		2	1	2	1
Grade 4			5	3	5
Febrile neutropenia grade 3					2
Nonneutropenic infection grade 3		1	1		
Platelets: grade 3			1		
Grade 4			1	1	
Anemia grade 3			1		1
Fatigue/↓Phosphate	1				
Fatigue grade 3					1
Hyperglycemia*			2		
Renal vein thrombus*			1		

*Probably unrelated.

For immunohistochemistry, 5-μm-thick formalin-fixed and paraffin-embedded tumor tissue sections were deparaffinized and hydrated. Sections were stained using mouse antibodies for Ki67 (monoclonal, clone MIB1; dilution, 1:200; 90-min incubation at room temperature; Dako, Inc.), CTR1 (polyclonal; dilution, 1:400; 90-min incubation at room temperature; Gene Tex, Inc.), DNMT1 (polyclonal; dilution, 1:100; 90-min incubation at room temperature; Santa Cruz Biotechnology), and p16 (monoclonal, clone JC8; dilution, 1:50; 60-min incubation at 37°C; Lab Vision Co.). As secondary antibody, Envision Plus Dual Link-labeled polymer (Dako, Inc.) was used. Apoptosis was studied using TUNEL assay (Promega Co.) according to manufacturer recommendations, but the diaminobenzidine reaction was stopped at 3 min.

Cytoplasmic CTR1, DNMT1, and p16 expression was quantified using a 4-value intensity score (0-3+). The cytoplasmic expression score (range, 0-300) was then obtained by multiplying the intensity score by the percent tumor cells staining. Nuclear CTR1, DNMT1, and Ki67 expression was reported as the percentage of positive nuclei among tumor cells assessed. For TUNEL assessment, we counted the number of positive apoptotic cells plus apoptotic bodies in 10 high power fields (×40; ref. 30). Changes in these scores and in number of mitoses per high power field, % necrosis, and apoptosis were calculated by subtracting day 1 values from day 12 values (baseline 0 values precluded calculation as % changes). Assessments were blinded with respect to drug dose, cohort, and % DNA methylation.

GraphPad Prism 5.0 was used for statistical calculations using two-tailed nonparametric tests (Spearman tests for correlations, Wilcoxon signed-rank tests for paired comparisons, and Mann-Whitney and Kruskal-Wallis tests for comparison of two groups or more than two groups, respectively). Small sample size precluded multivariate analyses. For dichotomization of continuous variables, cut-points were chosen arbitrarily by inspection of the data to try to maximize differences between higher and lower value groups.

Results

The trial accrued 31 patients from September, 2004 to March, 2007. Patient demographics are outlined in Table 1 and first-cycle toxicity in Table 2. First-cycle dose-limiting febrile neutropenia developed in two of six cohort five patients. Febrile neutropenia also developed during a later cycle of therapy in one patient in cohort 2. The dose recommended for phase II trials is 10 mg/m²/d days 1 to 5, and 8 to 12 or 15 mg/m²/d days 1 to 5 (with granulocyte colony-stimulating factor).

Time to progression and response. Median time to progression (TTP) was 7.1 (range, 4 to 29) weeks. There was one partial remission (thymoma). At first planned re-evaluation at 4 weeks, 17 of 28 evaluable patients had disease stability (including 3 minor responses in cutaneous T-cell lymphoma, malignant melanoma, and appendiceal adenocarcinoma, respectively). Tumor characteristics were similar in patients with partial or minor responses versus stability or progression. Fifteen patients received 1 decitabine cycle, 10 received 2, and 6 received 3 to 7 cycles. Tumor growth was the most frequent reason for therapy discontinuation.

Cohorts 4 to 5 had a shorter median TTP (4.5 versus 10 weeks; $P = 0.02$) and a trend toward greater increase in tumor size with first cycle (12.6% versus 7.0%; $P = 0.17$) than cohorts 1 to 3. Neither outcome correlated significantly with decitabine dose (r = -0.24 and r = 0.22, respectively) or with tumor characteristics, although there were slight trends toward TTP correlations with tumor type (median of 6.0 weeks for epithelial tumors versus 8.4 weeks for others; $P = 0.10$), number of mitoses (r = -0.34; $P = 0.09$), apoptosis score (r = 0.34; $P = 0.17$), and DNMT1 score (r = -0.32; $P = 0.12$) and toward tumor size change correlations with mitoses (r = 0.32; $P = 0.13$) and CTR1 score (r = 0.40; $P = 0.07$).

Time from last prior therapy and predecitabine tumor characteristics. Patients with shorter times from last prior cytotoxic or targeted therapies had a trend toward higher predecitabine tumor DNA methylation (55.2% for ≤ 3 months from last therapy versus 39.6% for > 3 months, $P = 0.07$; Fig. 1A). They also tended to have higher apoptosis scores (r = -0.44; $P = 0.07$) and had significantly higher p16 scores (Fig. 1B). Patients with last therapy ≤3 months before decitabine had lower predecitabine CTR1 scores than did those >3 months from last therapy (median score 90 versus 285; $P = 0.02$). CTR1 scores correlated more closely with time from last targeted or cytotoxic therapy (Fig. 1C) than with time only from last cytotoxic therapy (Fig. 1D), or time from last platinum (r = -0.13; $P = 0.63$). CTR1 score was not significantly different for patients who had versus had not received a platinum agent previously (median scores 100 versus 110; $P = 0.69$). Time from last therapy did not correlate with number of mitoses (r = 0.04), Ki67 positivity (r = -0.01), % necrosis (r = -0.09), or DNMT1 score (r = -0.009).

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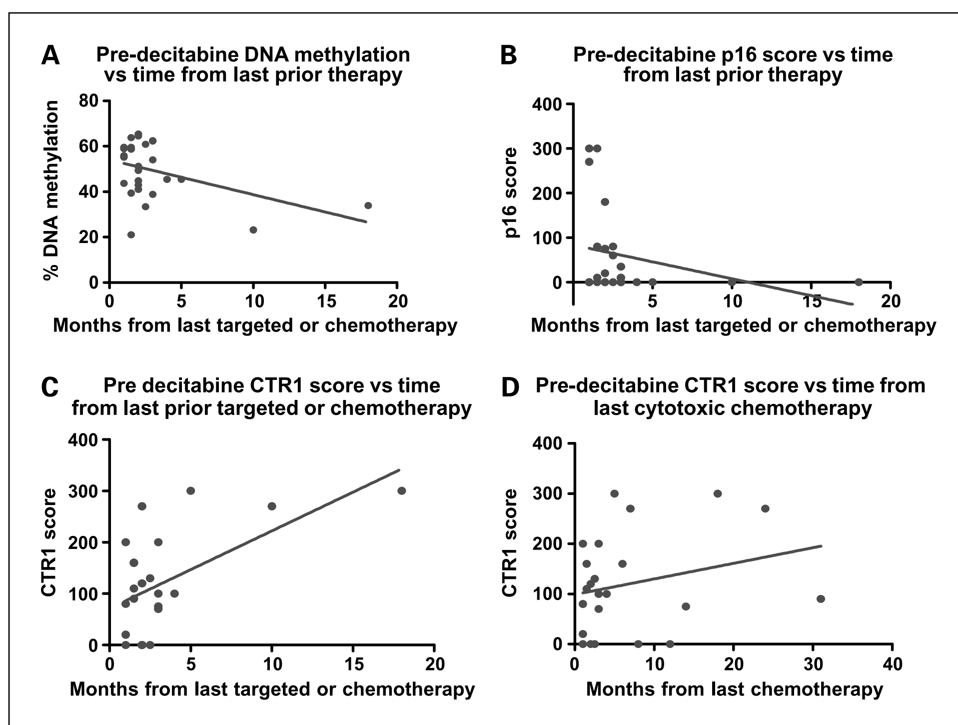

Fig. 1. Tumor characteristics and time from last prior therapy. *A,* predecitabine tumor DNA methylation versus time from last prior cytotoxic or targeted therapy (P = 0.07 for <3 versus >3 mo). *B,* predecitabine p16 score versus time from last prior cytotoxic or targeted therapy (r = −0.42; P = 0.04). *C,* predecitabine CTR1 score versus time from last prior cytotoxic or targeted therapy (r = 0.34; P = 0.11; P = 0.02 for >3 versus ≤3 mo). *D,* predecitabine CTR1 versus time from last prior cytotoxic chemotherapy (r = 0.22; P = 0.32).

Effect of decitabine on tumor global DNA methylation. In paired comparisons, there was a significant reduction in global tumor DNA methylation with decitabine, with a median relative reduction in tumor DNA methylation of 6% (mean, 8%; range, 52% decrease to 24% increase). Reduction in tumor DNA methylation was seen at all decitabine dose levels (median relative decreases 6%, 3%, 2.5%, and 12% with 25, 50, 75, and 100 mg/m^2/cycle, respectively; P = 0.16 for 100 mg/m^2/cycle versus others combined) and in all cohorts (median relative decreases 6%, 3%, 15%, 2.5%, and 4% in cohorts 1 to 5, respectively; P = 0.052 for cohort 3 versus others combined; Fig. 2A). Change in methylation did not correlate significantly with predecitabine tumor characteristics, although there tended to be greater reduction in methylation in tumors with more baseline mitoses (r = −0.40; P = 0.07).

Change in PBMC DNA methylation. PBMCs did not correlate significantly with tumors with respect to either DNA methylation (predecitabine and postdecitabine; r = 0.22; P = 0.09) or change in DNA methylation (r = 0.02; P = 0.91). Change in tumor DNA methylation was less than change in PBMC DNA methylation (PBMC median change, −14%; range, 47% reduction to 23% increase; P = 0.04 for tumor versus PBMC).

Gender and tumor type. Tumor DNA methylation was 52.5% in females versus 47.4% in males (P = 0.73), and methylation change was -8.4% versus -3.3% in females versus males (P = 0.39). "Standard" epithelial tumors tended to have slightly higher predecitabine methylation than did other tumor types (melanomas, lymphomas, thymomas, neuroendocrine carcinomas, desmoplastic tumors; 55.5% versus 45.4%; P = 0.13), and epithelial tumors had less DNA methylation change with decitabine (median reduction, 2.6% versus 11.2%; P = 0.026).

DNMT1 scores. Although cytoplasmic staining for DNMT1 was noted in 16 of 25 evaluable samples, nuclear staining was only evident in 8. Predecitabine, cytoplasmic DNMT1 scores did not correlate with DNA methylation (Table 3), and changes in these parameters with decitabine also did not correlate (r = -0.29). For tumors with predecitabine scores of >0, there was a reduction in cytoplasmic DNMT1 scores with decitabine (Table 4).

p16 scores. Predecitabine p16 score did not correlate with DNA methylation, but did correlate with apoptosis (Table 3), and was higher in epithelial tumors than in other types (median score, 75 versus 0; P = 0.01). Change in p16 score did not correlate with change in DNA methylation (r = 0.04), and decitabine had little effect on p16 scores (Table 4). Only 1 of 12 tumors initially negative for p16 converted to positive. Predecitabine *p16* promoter methylation was less than or equal to the background noise level (10%) in 25 of 28 evaluable tumors. The 3 with higher baseline levels went from 46% to 37%, 27% to 23%, and 16% to 47%, respectively, with p16 remaining undetectable in the first 2, and going from 10/300 to undetectable in the third.

CTR1 score. CTR1 staining was predominantly cytoplasmic, with nuclear staining identified in only six predecitabine and five postdecitabine samples. CTR1 scores were slightly lower in epithelial tumors than in other tumor types (median score, 80 versus 125; P = 0.15). Predecitabine CTR1 score was significantly higher in tumors with >7 versus ≤7 mitoses (Table 3). In paired comparisons, CTR1 scores increased significantly with decitabine when initial scores were <200 and in patients starting decitabine ≤3 months after last prior therapy (Table 4; Fig. 2B and C). CTR1 change did not vary significantly with dose or cohort. Although there was a strong inverse correlation between CTR1 score and methylation (Table 3; Fig. 2D), change in CTR1 score did not correlate significantly with change in methylation (r = 0.23), and *CTR1* promoter methylation was <10% (background noise level) in

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all evaluable tumor samples (24 predecitabine, 26 postdecitabine) and in all 36 cell lines tested.

DNA methylation and markers of proliferation and cell death, tumor size change, and TTP. Mitoses and Ki67 tended to *increase* with decitabine in tumors in which they were initially low and apoptosis increased significantly, but necrosis did not change (Table 4). Predecitabine DNA methylation did not correlate with mitoses, Ki67, % necrosis, or apoptosis (Table 3), and also did not correlate with tumor size change over first therapy cycle (r = -0.20) or with TTP (r = 0.20). Similarly, change in DNA methylation did not correlate significantly with change in any of mitoses (r = 0.07), Ki67 (r = 0.16), apoptosis (r = 0.002), necrosis (r = -0.38; *P* = 0.08), or tumor size (r = -0.26) or with TTP (r = 0.29; *P* = 0.20). Despite the lack of correlation of predecitabine methylation and methylation change with these factors, postdecitabine methylation did correlate with postdecitabine mitoses (r = -0.56; *P* = 0.002) and Ki67 (r = -0.43; *P* = 0.04), with a trend to an association with TTP (r = 0.29; *P* = 0.15).

Blood pressure and pH factors. Patients with SBP <120 versus ≥120 mm Hg had significantly higher predecitabine tumor necrosis (median, 40% versus 15%; *P* = 0.03). For SBP <140 versus ≥140 mm Hg, first cycle tumor size change was 7.5% versus 15.6% (*P* = 0.03), whereas DNA methylation change was -3.3% versus -11.6% (*P* = 0.10). If SBP is multiplied by dose/cycle (because both higher SBP and higher dose might increase tumor exposure to drug), DNA methylation change was -12% versus -3% for patients with values ≥9,000 versus <9,000 (*P* = 0.04). There was a trend toward a greater reduction in DNA methylation with higher predecitabine serum LDH (r = -0.38; *P* = 0.058), possibly because LDH also correlated with predecitabine mitoses (r = 0.40; *P* = 0.04). There were no correlations of interest between serum glucose, chloride or CO_2, and changes in DNA methylation.

Discussion

This daily ×5 to 10 decitabine regimen was well tolerated in very heavily pretreated solid tumor and lymphoma patients. Neutropenia was dose limiting. Decitabine reduced global DNA methylation, particularly in nonepithelial tumors in this study. In leukemias, dose-response curves for decitabine-induced demethylation flatten at higher doses (33). Although the relationship between dose and effect was not statistically significant in our study, trends to increased demethylation with both higher decitabine doses and with higher SBP [which might augment tumor blood flow (23, 24), thereby enhancing drug delivery; ref. 22] suggest a dose-response effect, as previously suggested for 6-hour decitabine infusions in solid tumors (16). However, methylation decreased even with lowest doses tested, and the changes we noted at lower doses were comparable with those previously reported at higher doses using 6-hour decitabine infusions (16).

In our study, we saw a median relative reduction in tumor DNA methylation of 6% (mean, 8%; range, 52% decrease to 24% increase), with a median 12% decrease in methylation at the highest dose tested (100 mg/m^2/cycle). In comparison, using a single 6-hour i.v. infusion of decitabine in combination with carboplatin, Appleton et al. (16) noted a mean demethylation of the *MAGE1A* promoter of 3.5% in tumor at their decitabine maximum tolerated dose of 90 mg/m^2/cycle, with a maximal demethylation of 6.8%. Although we reported a relative change in % DNA methylation, it was not clear whether Appleton et al. (16) were reporting a relative or an absolute change in methylation. Aparicio et al. (34) administered decitabine 20 to 40 mg/m^2 to solid tumor patients as a 72-h continuous i.v. infusion, and detected decreased promoter methylation for some genes in some patients, but they did not report the degree of demethylation. Furthermore, in their

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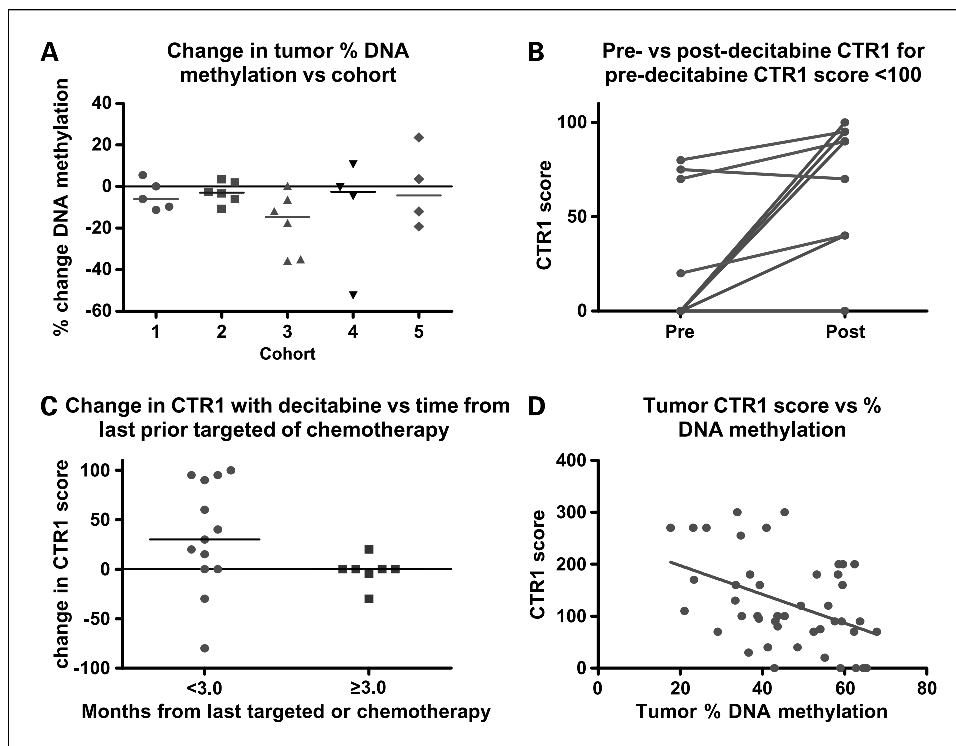

Fig. 2. Change in tumor characteristics with decitabine. *A,* change in tumor % DNA methylation versus cohort (across all cohorts, *P* = 0.40; cohort 3 versus combined others: median, –15% versus –3%; *P* = 0.052). *B,* predecitabine versus postdecitabine CTR1 score for predecitabine CTR1 of <100 (median, 0 versus 90; paired *P* = 0.02, increase in 8 of 10). *C,* change in CTR1 score with decitabine for patients with last prior therapy <3 versus ≥3 mo before decitabine (median, 30 versus 0; *P* = 0.03). *D,* tumor CTR1 score versus % DNA methylation predecitabine and postdecitabine (r = –0.41; *P* = 0.005).

study, methylation was instead increased in some patients, and change in methylation did not correlate with decitabine dose (34). Schrump et al. (35) also gave decitabine 60 to 90 mg/m^2 as a continuous i.v. infusion to solid tumor patients, and postdecitabine, they noted increased expression of 75 genes but decreased expression of 324 genes, and they did not report % change in DNA methylation. Overall, the available data from our study and others suggests that decitabine is able to reduce DNA methylation in solid tumors. The effect of dose is unclear, but our data and Appleton's data suggest that higher doses within the range tolerated may have a greater effect. The effect of schedule of drug administration is also unclear. There is no indication that our daily 1-h infusion schedule was any less effective than more prolonged administration schedules.

PBMC DNA methylation was not a reliable surrogate for tumor methylation. We and others (16) found a greater decitabine effect on methylation in PBMCs than in tumors, possibly due to differences in kinetics or drug accessibility. DNA synthesis is required for decitabine incorporation into DNA, for DNMT entrapment and for DNA demethylation (36), in keeping with the trend noted toward greater demethylation in tumors with more predecitabine mitoses.

Decitabine promotes proteosomal degradation of DNMT (18), and DNMT1 was decreased in tumors in which it was detectable predecitabine by our immunohistochemistry methods, although changes in DNMT1 and methylation did not correlate. The previously reported proteosomal degradation of DNMT1 in cell lines was seen predominantly in the cell nucleus (18), although we found mainly cytoplasmic changes in DNMT1 in our study. We are unaware of any other clinical assessments of effect of decitabine on DNMT1 expression, by immunohistochemistry. Because the functional role of DNMT1 is within the cell nucleus, it is unclear whether the changes in cytoplasmic expression of DNMT1 we detected are of any biological significance.

In secondary exploratory analyses (which should be interpreted cautiously in light of small patient numbers, population heterogeneity, multiplicity of analyses, and use of semiquantitative immunohistochemistry), mitoses and Ki67 tended to increase with decitabine when initially low, postdecitabine mitoses, and Ki67 correlated inversely with postdecitabine methylation, and TTP tended to be shorter with low postdecitabine methylation, suggesting that, although decitabine increases apoptosis, it may also enhance proliferation by up-regulating pro-growth signaling pathways. The association of shorter TTP with schedule could possibly indicate that effect on proliferation varies with decitabine schedule, although patient selection might also account for this. Hence, decitabine might be better used in combination with other agents as a potential resistance modulator rather than being used alone in solid tumors.

Platinum-resistant cell lines may have reduced CTR1 (a copper transporter that plays a role in cellular platinum uptake; ref. 37) and multiple other membrane transporters (9, 10), and decitabine may up-regulate some transporters in platinum-resistant cells (9). We hypothesized that the dose-response curve flattening seen at higher chemotherapy doses in non–small cell lung cancer and other malignancies could be explained in part by down-regulation and saturation of factors required for drug efficacy, including various transporters (7). Here, we found that, compared with tumors not recently treated, tumors treated recently with any cytotoxic or targeted therapy had significantly less CTR1 but increased p16 and trends to increased methylation and apoptosis. Although *CTR1* promoter was not hypermethylated, there was a strong negative correlation between global DNA methylation and CTR1 score, and administration of decitabine (which activates gene expression through mechanisms both dependent on and independent of promoter hypomethylation; refs. 2, 4) significantly increased CTR1 score for those with initial scores of <200 and for patients who had received their last prior therapy <3 months earlier. Hence, DNA hypermethylation may play an indirect role in decreasing CTR1 expression (for example, by decreasing cell proliferation), and decitabine may be effective at increasing its expression by up-regulating expression of factors that in turn promote CTR1 expression. We are currently also assessing expression of other transporters in these tumors. Alternatively, it remains possible that the increase in CTR1 is a more nonspecific effect of chemotherapy administration and that agents with other mechanisms of action would also increase CTR1 expression. Against this possibility is the observation that CTR1 expression increased with increasing time from last therapy with other agents.

In keeping with published cell line (9) and xenograft (8) data, our observations suggest a potential role for decitabine as a resistance modulator in tumors with reduced transporters. Although other dose-schedules have been ineffective (38, 39), combining multiple day decitabine administration with platinums in chemonaive patients could be of interest. DNA synthesis (during either cell division or DNA repair) is

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Table 3. Spearman coefficients for correlations between predecitabine tumor characteristics

	n	P16 score	CTR1 score	DNMT1 score	Tumor % DNA methylation	Apoptosis score	% necrosis	% Ki67 positive
No. of Mitoses	26	−0.14	0.39* (P = 0.06)	0.17	0.004	−0.13	0.17	0.14
Ki67% positive	23	0.26	0.19	0.04	0.07	−0.04	−0.36 (P = 0.09)	
% necrosis	27	−0.22	−0.08	−0.25	−0.20	−0.18		
Apoptosis score	18	0.53 (P = 0.03)	0.03	−0.21	0.12			
Tumor % DNA methylation	27	0.21	−0.45 (P = 0.04)	−0.15				
DNMT1 score	25	0.02	0.34 (P = 0.11)					
CTR1 score	23	0.05						
P16 score	25							

NOTE: Only P values <0.20 are shown.
*Predecitabine CTR1 score was significantly higher in tumors with >7 mitoses than in those with ≤7 (200 vs 85, P = 0.02).

Table 4. Postdecitabine vs predecitabine tumor characteristics

Factor	n*	Predecitabine		Postdecitabine		Wilcoxon signed rank (paired) P
		Median	Range	Median	Range	
No. of mitoses:						
All	24	2.0	0-15	3.5	0-28	0.12
If predecitabine mitoses ≤7	18	1.5	0-7	3.0	0-28	0.02
% tumor cells expressing Ki67:						
All	20	20	0-70	22.5	0-85	0.53
If predecitabine Ki67 ≤25	14	7.5	5-25	12.5	5-75	0.15
% of tumor that is necrotic:						
All	25	20	0-90	20	0-70	0.32
If predecitabine necrosis >30%	8	65	40-90	35	0-70	0.02
If predecitabine necrosis ≤30%	17	5	0-30	5	0-50	0.30
Apoptosis score	17	22	2-230	49	0-269	0.049
% DNA methylated:						
Tumor	25	51.2	21.1-65.4	43.7	17.7-67.9	0.01
Blood	30	65.3	39.5-74.1	56.0	33.8-64.5	<0.001
DNMT1 cytoplasmic score:						
All	23	30	0-280	50	0-180	0.56
If predecitabine DNMT1 >0	16	120	20-280	95	0-180	0.04
DNMT1 nuclear score						
All	23	0	0-110	0	0-40	0.50
If predecitabine DNMT1 >0	7	10	5-110	5	0-40	0.50
CTR1 score:						
All	21	100	0-300	95	0-300	0.22
If predecitabine CTR1 <200	15	75	0-160	90	0-255	0.025
If predecitabine CTR1 <100	9	0	0-80	90	0-100	0.02
If ≤ 3 months from last therapy	17	80	0-270	95	0-270	0.04
P16 score	24	5	0-300	0	0-300	0.96
% of tumor that is stroma	26	40	0-90	50	1-90	0.97
% of tumor that is fibrosis	26	30	0-60	35	0-80	0.49

*Number for which both the predecitabine and postdecitabine value is known and the predecitabine value satisfies any criteria specified in left column.

required for decitabine-induced hypomethylation (36). Platinum binding to DNA generates DNA repair (6). Hence, platinums could potentiate DNA demethylation by augmenting decitabine incorporation into DNA, whereas DNA demethylation could potentially inhibit emergence of resistance to the platinum.

Decitabine may also augment epidermal growth factor receptor (EGFR) expression and restore sensitivity to EGFR inhibitors (40), suggesting a role for decitabine in reversing some types of acquired resistance to EGFR inhibitors. Furthermore, our observation here that CTR1 expression may be reduced by recent exposure to targeted therapies may help explain why addition of small molecule EGFR inhibitors to chemotherapy in non–small cell lung cancer adds little (41, 42), whereas addition of anti-EGFR antibodies to chemotherapy may improve outcome (43). Cellular uptake of small molecules could hypothetically be reduced by down-regulation of membrane transporters, whereas antibodies would not require cellular uptake.

Correlation of low SBP with increased tumor necrosis is in keeping with tumor blood flow being particularly sensitive to SBP (23, 24). The additional observations that high SBP correlated with greater tumor growth with first decitabine cycle, but decitabine-induced demethylation was greater with increased SBP (possibly through improved drug delivery) suggest testing of a strategy to maintain SBP at low levels between chemotherapy cycles but to adjust medications to promote high SBP during chemotherapy administration and distribution.

Although the effect of decitabine on DNA methylation and other parameters was modest, our data support further exploration of decitabine as a resistance-modulating agent. Patients most likely to benefit may be those most recently treated with other agents and those with lowest expression of drug transporters.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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