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ABSTRACT

Resistance to Imatinib mesylate is emerging as a real clinical problem in the management of chronic myelogenous leukemia (CML). In this project, we are exploring the hypothesis that epigenetic silencing associated with promoter DNA methylation mediates resistance in selected cases, and that reversal of silencing by decitabine-induced hypomethylation can be of therapeutic benefit in CML. In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified methylation of p15 and CDH13 in subsets of patients but ruled these genes out as major causes of resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML.

Table of Contents

Cover.....	
SF 298.....	
Introduction.....	Page 4
Body.....	Page 4-10
Key Research Accomplishments.....	Page 10
Reportable Outcomes.....	Page 10
Conclusions.....	Page 10
References.....	Page 11
Appendices.....	Page 12-21

Introduction

Abstract

Resistance to Imatinib mesylate is emerging as a real clinical problem in the management of chronic myelogenous leukemia (CML). In this project, we are exploring the hypothesis that epigenetic silencing associated with promoter DNA methylation mediates resistance in selected cases, and that reversal of silencing by decitabine-induced hypomethylation can be of therapeutic benefit in CML. In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified methylation of p15 and CDH13 in subsets of patients but ruled these genes out as major causes of resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML.

Subject Terms

Chronic myelogenous leukemia, epigenetics, DNA methylation, decitabine, imatinib, clinical trial

Body

Tasks

Task 1. Determine the methylation profile of candidate tumor-suppressor genes in CML patients treated with Imatinib, months 1-24:

- a. Identify in the leukemia database all patients treated with Imatinib at MDACC for whom follow-up of over 1 year is available (month 1)
- b. Collect paraffin-embedded pre-treated bone marrow biopsies on all patients (projected 400 patients, 40 cut/month, months 1-10)
- c. Extract DNA from paraffin cuts (start month 1 – ongoing until all samples collected, months 1-10)
- d. Bisulfite treatment and PCR-based methylation analysis for all the genes (months 2-20)
- e. Statistical analysis of the collected data (months 21-22)
- f. Validation of the results on prospectively collected samples (months 23-36)

Task 2. Conduct a clinical trial of 5-aza-deoxycytidine followed by Imatinib in patients with CML resistant to, or less likely to respond to Imatinib.

- a. Treat initial 6 patients for toxicity analysis of the combination (months 1-2)
- b. Enroll patients on the phase II study (projected 3-5/month, months 1-24)
- c. Continue subsequent cycles and follow-up of the patients (months 25-36)

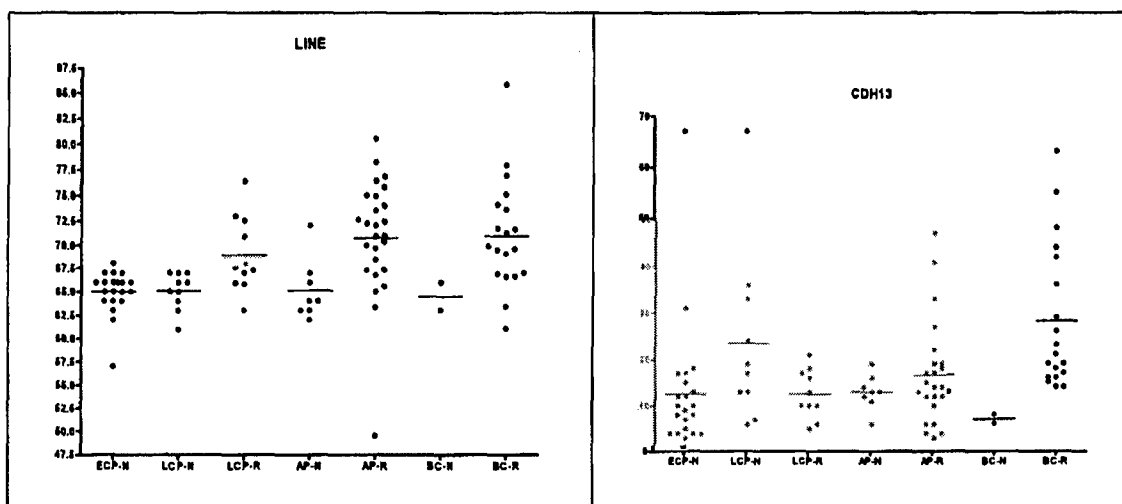
Task 3. Determine the methylation and expression status of candidate tumor-suppressor genes after treatment with 5-aza-deoxycytidine and correlate these values with subsequent responses to Imatinib.

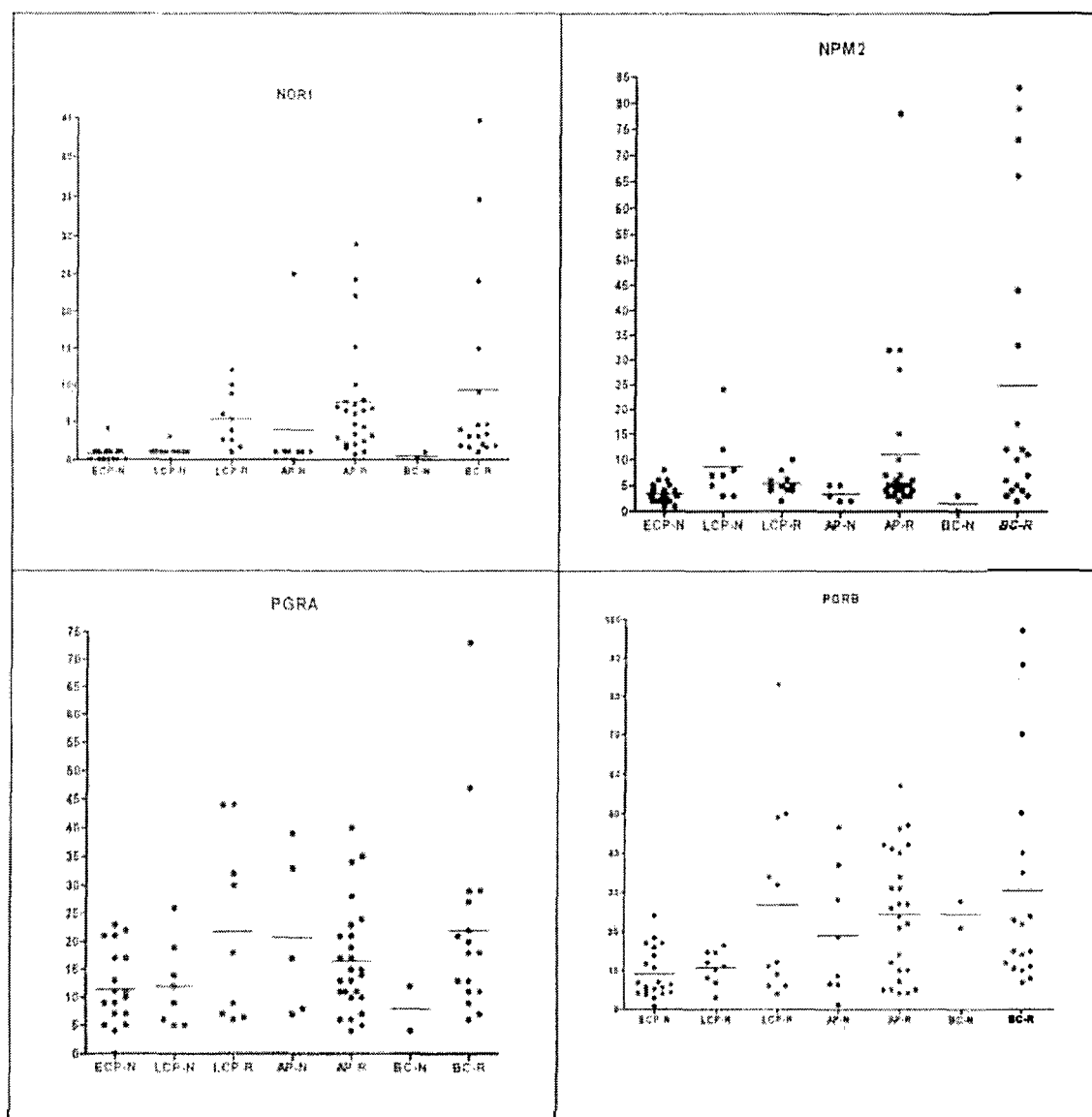
- Collect samples before and after treatment (months 1-24)
- Analyze samples for methylation (months 13-36)
- Analyze samples for gene expression (months 13-36)
- Statistical analysis of the collected data (months 32-36)

Progress on Task 1:

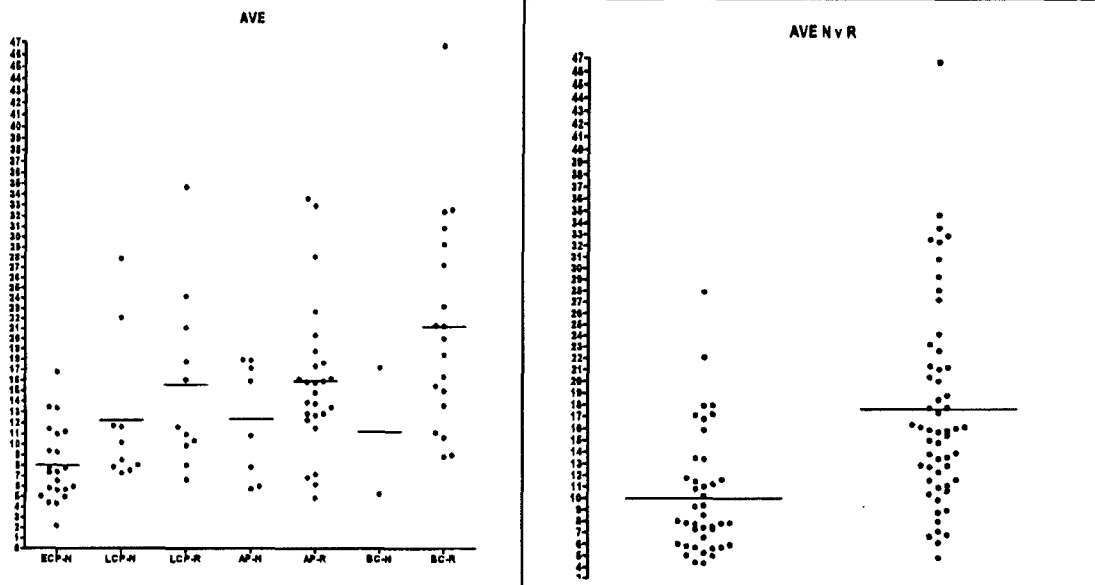
We have obtained samples from 200 patients with CML at various phases with outcome data and have extracted DNA from them (Tasks 1a-1c). A pilot study of multiple genes has identified a panel of genes (P15, LINE, PGRB, CDH13, NOR1, NPM2, DPYS, RIL) that are informative for DNA methylation in CML. We are proceeding with bisulfite/pyrosequencing analysis of all these samples (Task 1d) and statistical analysis (Task 1e). These tasks have not been completed yet but are well underway. Preliminary data shown that DNA methylation increases with disease progression but, more importantly, increases substantially in patients who become resistant to Imatinib, confirming the original hypotheses.

The following graphs show examples of the data. ECP=Early chronic phase; LCP=Late chronic phase; AP=Accelerated phase; BC=Blastic crisis; N=Imatinib naïve; R=Imatinib resistant. Each dot is a patient sample. On the Y axis is % methylation. Gene name is on top of each graph.





The data were then analyzed by deriving an average of all genes and comparing the average (1) across the different categories and (2) simply by Imatinib sensitive/resistant. These graphs are shown next.



There was a statistically higher degree of methylation in Imatinib resistant cases (2-sided $p < 0.001$, Mann-Whitney test).

Thus, to summarize progress for this task, we have successfully identified and retrieved samples from patients with CML treatment naïve or resistant to Imatinib, have extracted DNA from those and initiated methylation studies. Preliminary analyses suggest significantly higher methylation levels in Imatinib resistant cases, thus confirming our hypothesis. We are studying additional cases and will next move on to Task 1f.

Progress on tasks 2 and 3

At the outset of the grant, tasks 2 and 3 were modified slightly to include only analysis of samples collected as part of the clinical trials outlined.

A clinical trial of single agent decitabine was performed in Imatinib-resistant CML. This trial included correlative studies funded by this grant and it was recently published¹. The abstract follows:

Purpose. To determine the activity of decitabine, a DNA methylation inhibitor, in imatinib refractory or intolerant chronic myelogenous leukemia (CML).

Patients and methods. Thirty five patients were enrolled in this phase II study (12 in chronic phase [CP], 17 in accelerated phase [AP] and 6 in blastic phase [BP]). Decitabine was administered at 15 mg/m² IV over one hour daily, 5 days a week for two weeks. DNA methylation was measured using a LINE1 bisulfite/pyrosequencing assay.

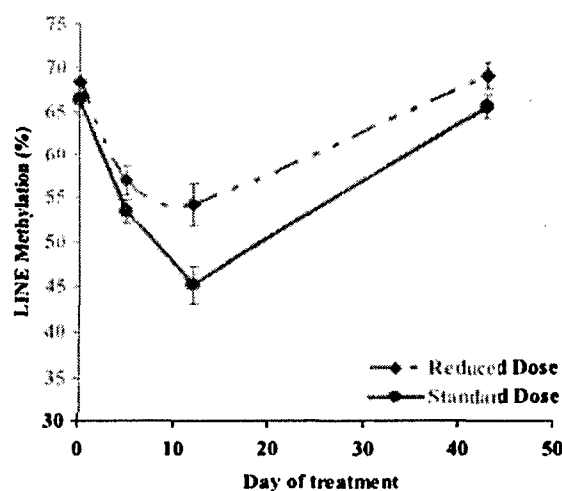
Results. Complete hematologic responses were seen in 12 patients (34%) and partial hematologic responses in 7 patients (20%), for an overall hematologic response rate of 54% (83% in CP, 41%

in AP and 34% in BP). Major cytogenetic responses were observed in 6 patients (17%), and minor cytogenetic responses were seen in 10 patients (29%) for an overall cytogenetic response rate of 46%. Median response duration was 3.5 months (range 2-13+ months). Myelosuppression was the major side-effect, with neutropenic fever in 28/124 (23%) courses of therapy. LINE1 methylation decreased from $71.3 \pm 1.4\%$ (mean $\pm$ SEM) to $60.7 \pm 1.4\%$ after 1 week, $50.9 \pm 2.4\%$ after 2 weeks and returned to $66.5 \pm 2.7\%$ at recovery of counts (median, 46 days). LINE1 methylation at the end of week 1 did not correlate with subsequent responses. However, at day 12, the absolute decrease in methylation was $14.5 \pm 3.0\%$ vs. $26.8 \pm 2.7\%$ in responders vs. non-responders ($p=0.007$).

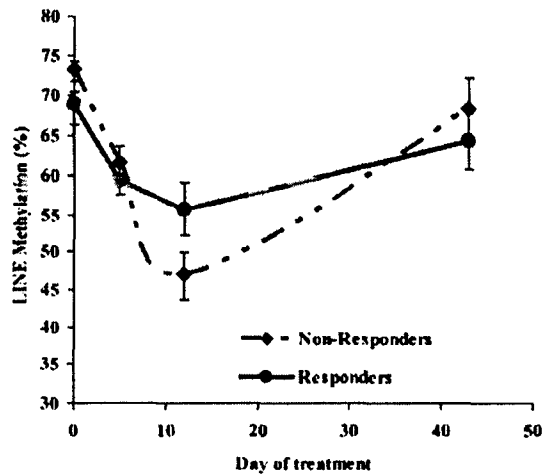
Conclusions. Decitabine induces hypomethylation and has clinical activity in imatinib refractory CML. We hypothesize that the inverse correlation between hypomethylation and response is due to a cell death mechanism of response, whereby resistant cells can withstand more hypomethylation.

Highlights of the correlative studies were:

(1) Methylation decreases in treated patients in a dose-dependent way:



(2) Methylation decreases more in non-responders at 10 days, consistent with a cell death mechanism of action of the drug, whereby non-responders have hypomethylation but do not die.



Another study was initiated that combined Imatinib with Decitabine. This study has accrued well and is summarized below:

The objective of this phase II study is to investigate the activity of decitabine in combination with imatinib mesylate in patients with Ph-positive CML-AP or non-lymphoid CML-BP. To be eligible to this study, patients who had previously been treated with imatinib must have evidence of imatinib failure. Treatment schedule was imatinib 600mg daily and decitabine intravenously at 15mg /m² x 5 days/week for two consecutive weeks, and two week off. Two courses were planned for all the patients, and patients were required to receive at least 2 courses of treatment to be evaluable for response analysis. From January 2003 to July 2005, 27 patients (8 BP, 19 AP) were enrolled into this study. Clonal evolution was observed in 21 patients (77%). Twenty-four patients (88%) were previously treated with imatinib. Median bone marrow blast percentage was 11% (range 0%-91%). All the patients received at least 1 cycle of the treatment (median 3, range 1-12). Toxicity was evaluated in all patients; grade 3/4 toxicity included infection (n=9), CNS bleed (n=2), GI bleed (n=2), dyspnea (n=1), diarrhea (n=1), and edema (n=1). Seven patients (26%) received only 1 cycle of treatment, owing to early death from acute disease progression (n=5), recurrent subdural hematoma (n=1), and a patient's decision due to grade 3 diarrhea (n=1). Twenty patients (74%) received 2 or more treatment cycles and were evaluable for response. Nine patients (45%) achieved complete hematologic response (CHR: BM blast <5%, normalization of peripheral blood and disappearance of signs and symptoms of the disease), and 3 patients (15%) achieved partial hematologic response (PHR: same criteria except for persistence of immature cells, splenomegaly <50% or thrombocytosis >450x10⁹/L but <50% of pretreatment). Among patients with CHR, 7 patients (35%) achieved cytogenetic response (3 complete cytogenetic response (Ph 0%), 1 partial cytogenetic response (Ph<35%), and 3 minor cytogenetic response (Ph<90%)). Median response duration was 13 weeks (range 4-107+). Four patients (15%) who achieved CHR eventually underwent allogeneic stem cell transplantation. Median overall survival of all patients with AP and BP were similar in this study (49 and 53 weeks, respectively). Median overall survival of patients with cytogenetic response (n=7), hematologic response only (n=5), and non-responders (n=8) were 86, 63, 19 weeks, respectively

($p < 0.01$). In summary, decitabine in combination with imatinib mesylate is an active regimen for CML-AP and BP, even in patients previously treated with imatinib. Correlative laboratory analysis and further patients accrual is ongoing.

Key research accomplishments

- Identified samples from patients with CML suitable for analysis of mechanisms of resistance to imatinib
- Determined that methylation increases with CML progression, and is increased further at Imatinib resistance
- Analyzed samples from patients treated with decitabine in a phase 1 study and showed dose dependent hypomethylation in-vivo
- Analyzed samples from patients with imatinib resistant CML treated with decitabine, confirmed hypomethylation in-vivo, found correlations between degree of methylation at 10 days after therapy and lack of response and found similar hypomethylation at the development of resistance to decitabine in CML, suggesting a non-pharmacologic mechanism of resistance
- Collected samples from patients with imatinib resistant CML treated with a combination of imatinib and decitabine

Reportable outcomes

Manuscript published (attached in the appendix).

Issa, J.P., Gharibyan, V., Cortes, J., Jelinek, J., Morris, G., Verstovsek, S., Talpaz, M., Garcia-Manero, G. & Kantarjian, H.M. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. *J. Clin. Oncol.* 23, 3948-3956 (2005).

Conclusions

In progress to date, we have identified samples from patients with CML prior to Imatinib therapy, as well as from patients with established resistance to Imatinib. Bisulfite based analysis identified increased methylation in patients resistant to Imatinib, suggesting the hypothesis that critical gene silencing contributes to this resistance. In parallel, clinical trials of decitabine have shown activity as single agent and when combined with Imatinib in CML resistant to Imatinib. Analysis of samples from patients on trial showed hypomethylation after therapy. Hypomethylation dynamics suggest that decitabine leads to CML cell death 5-10 days after treatment and suggest that resistance to decitabine is not pharmacologic. These studies are ongoing to clarify the role of methylation in the pathogenesis and therapy of Imatinib resistant CML. "So what": (1) Methylation analysis shows association with Imatinib resistance. Predictive value of such tests will be studied next; (2) The hypomethylating drug decitabine has clinical activity in imatinib resistant CML, and analysis of CML samples after therapy may predict response to this agent.

References

Reference List

1. Issa, J.P., Gharibyan, V., Cortes, J., Jelinek, J., Morris, G., Verstovsek, S., Talpaz, M., Garcia-Manero, G. & Kantarjian, H.M. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. *J. Clin. Oncol.* **23**, 3948-3956 (2005).

Appendix:

Phase II Study of Low-Dose Decitabine in Patients With Chronic Myelogenous Leukemia Resistant to Imatinib Mesylate

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose

To determine the activity of decitabine, a DNA methylation inhibitor, in imatinib-refractory or intolerant chronic myelogenous leukemia.

Materials and Methods

Thirty-five patients were enrolled in this phase II study (12 in chronic phase, 17 in accelerated phase, and six in blastic phase). Decitabine was administered at 15 mg/m² intravenously over 1 hour daily, 5 days a week for 2 weeks. DNA methylation was measured using a LINE1 bisulfite/pyrosequencing assay.

Results

Complete hematologic responses were seen in 12 patients (34%) and partial hematologic responses in seven patients (20%), for an overall hematologic response rate of 54% (83% in chronic phase, 41% in accelerated phase, and 34% in blastic phase). Major cytogenetic responses were observed in six patients (17%), and minor cytogenetic responses were seen in 10 patients (29%) for an overall cytogenetic response rate of 46%. Median response duration was 3.5 months (range, 2 to 13+ months). Myelosuppression was the major adverse effect, with neutropenic fever in 28 (23%) of 124 courses of therapy. LINE1 methylation decreased from 71.3% ± 1.4% (mean ± standard error of the mean) to 60.7% ± 1.4% after 1 week, 50.9% ± 2.4% after 2 weeks, and returned to 66.5% ± 2.7% at recovery of counts (median, 46 days). LINE1 methylation at the end of week 1 did not correlate with subsequent responses. However, at day 12, the absolute decrease in methylation was 14.5% ± 3.0% versus 26.8% ± 2.7% in responders versus nonresponders (*P* = .007).

Conclusion

Decitabine induces hypomethylation and has clinical activity in imatinib refractory chronic myelogenous leukemia. We hypothesize that the inverse correlation between hypomethylation 2 weeks after therapy and response is due to a cell death mechanism of response, whereby resistant cells can withstand more hypomethylation.

J Clin Oncol 23. © 2005 by American Society of Clinical Oncology

INTRODUCTION

Decitabine (5-aza-2'-deoxycytidine) is a cytosine analog that incorporates into DNA and depletes DNA methyltransferase protein levels, resulting in replication-dependent DNA hypomethylation.^{1,2} In vitro, this hypomethylation is associated with reactivation of multiple genes, including tumor-suppressor genes,³ and it is thought

that this effect on gene expression contributes to the mechanism of responding to the drug. At high doses, decitabine induces DNA adducts that eventually result in cytotoxicity.⁴ Decitabine has single-agent activity in myeloid malignancies^{1,5} including acute myelogenous leukemia, chronic myelogenous leukemia (CML), and the myelodysplastic syndromes. A recent study

established that decitabine is equally (or perhaps more) effective at relatively low doses compared with high doses.⁶ A dose of 15 mg/m² intravenously (IV) over 1 hour daily 5 days per week for 2 consecutive weeks was recommended for further phase II studies. The hypomethylating effect of decitabine was measured by various techniques including measurement of global methylation by liquid chromatography mass spectrometry, measurement of repetitive element methylation by bisulfite/pyrosequencing, and measurement of individual gene methylation by various methods (Yang et al, manuscript submitted for publication). In general, the methods measuring global methylation gave equivalent results, and demonstrated a dose-dependent decrease in methylation, with a plateau at 150 to 200 mg/m² cumulative dose.

The treatment of CML was revolutionized by the tyrosine kinase inhibitor imatinib mesylate (imatinib).⁷ All phases of CML respond to this drug,⁸⁻¹⁰ and it is now recommended for front-line therapy of this disease. However, resistance to imatinib is increasingly recognized as a clinical problem, particularly in blastic phase (BP), where it is nearly universal, and in accelerated phase (AP), where it is common as well.^{11,12} A number of reports have also documented the emergence of imatinib resistance in a subset of patients with chronic phase (CP) CML.¹³ Resistance to imatinib can be attributed to mutations in the *BCR/ABL* gene in about 30% to 50% of all cases.^{12,14} The prognosis of patients who develop imatinib resistance is poor.¹³ Given that decitabine and imatinib have distinct mechanisms of action, we reasoned that decitabine, which had demonstrated activity in CML before the imatinib era,¹⁵ might also be active in imatinib-resistant CML.

Here, we report the results of a phase II study of decitabine in CML showing hematologic and cytogenetic responses in all phases of the disease. In addition, we show that decitabine therapy is associated with hypomethylation; suggest the hypothesis that responses are related to induction of cell death by hypomethylation; and provide evidence that resistance to decitabine in CML is not pharmacologic, but is possibly related to resistance to hypomethylation-induced cell death.

MATERIALS AND METHODS

Study Group

Patients entered onto the study were required to have a diagnosis of Philadelphia chromosome (Ph)-positive CML and to have evidence of resistance or intolerance to imatinib. Resistance to imatinib was defined by elevated WBC or platelet counts while on therapy, lack of any cytogenetic response after 12 months of therapy (Ph, 100%), appearance of accelerated or blastic phase features while on therapy, reappearance of the Ph-positive clones after an initial complete cytogenetic response to imatinib, or an increase in Ph-positive cells by 30% or more while on therapy. Intolerance to imatinib was defined by the development of life-threatening adverse effects requiring discontinuation of therapy.

Other eligibility criteria were adequate performance status (Eastern Cooperative Oncology Group performance status ≥ 2) and adequate cardiac (New York Heart Association class III-IV excluded) and hepatorenal functions (creatinine < 2 mg/dL; bilirubin < 2 mg/dL, and hepatic enzymes $< 2 \times$ upper limit of normal). All patients gave written informed consent indicating that they were aware of the investigational nature of the study, in keeping with the policies of the M. D. Anderson Cancer Center (Houston, TX). Consent was also obtained for the (optional) correlative studies, which included the collection of additional blood samples.

Treatment

The initial plan was to treat patients at 15 mg/m² daily IV over 1 hour for 10 days (5 days on, 2 days off, 5 days on), approximately every 6 weeks as indicated by follow-up counts and marrow studies. After the first 10 patients with CML-CP were treated, the initial dose was reduced to 10 mg/m² daily IV over 1 hour for 10 days (5 days on, 2 days off, 5 days on) because of myelosuppression. The starting dose was not changed for patients with AP or BP. After the first cycle, patients were dose-adjusted for toxicity. If $\geq$ grade 3 nonhematologic toxicity was felt attributable to the drug, the patients were taken off-study. Patients experiencing grade 3 or worse hematologic toxicity were evaluated for persistent disease. If myelosuppression was attributed to disease, the treatment was continued at the same dose level. If myelosuppression was attributed to the drug, subsequent cycles were given at a dose that was lowered by 5 mg/m²/d of treatment. The use of hydroxyurea was allowed for the first two cycles of therapy, at the discretion of the investigators. Prophylactic antibiotics were recommended for all patients who became neutropenic. Growth factors were not routinely used, but were allowed in patients who developed fever and neutropenia. In general, patients were treated every 6 weeks for two cycles, and treatment was continued beyond two cycles unless clear evidence of lack of response or disease progression was observed.

Response Criteria and Statistical Considerations

Response criteria were as previously described.^{15,16} A complete hematologic response (CHR) required disappearance of all signs and symptoms related to disease, normalization of peripheral counts (absolute neutrophil count $\geq 10^9$ /L, platelet count $\geq 100 \times 10^9$ /L), and a normal bone marrow morphology with $\leq 5\%$ marrow blasts. A partial hematologic response was defined as a CHR, but with persistence of peripheral immature cells ($< 5\%$ myelocytes + metamyelocytes), or persistent splenomegaly or thrombocytosis that were reduced by 50% or more from pretreatment levels. A hematologic improvement was defined as a return to a second CP, referring to disappearance of accelerated-BP criteria. Cytogenetic responses were measured separately by G-banding in at least 20 metaphases and were categorized by the degree of suppression of Ph-positive cells as a complete cytogenetic response (Ph, 0%), partial cytogenetic response (Ph, 1% to 34%), or minor cytogenetic response (Ph, 35% to 95%). Remission was calculated from date of first response until relapse. Survival was calculated from start of therapy until death from any cause.

Analysis of DNA Methylation

Peripheral blood was collected from consenting patients treated as part of this protocol. Whenever possible, blood was obtained before or on the first day of treatment (day 0 or 1), at the end of the first week or at the beginning of the second week (days

Decitabine in CML

5 to 7), at the end of the second week (day 12), and at recovery of counts. DNA was isolated from peripheral-blood samples after ficoll separation of mononuclear cells using standard phenol-chloroform extraction methods. After extraction, DNA was modified with sodium bisulfite. This induces deamination of unmethylated cytosines converting unmethylated CpG sites to UpG without modifying methylated sites. Bisulfite treatment of genomic DNA was performed as described.¹⁷ Two μ g of DNA were used. DNA was denatured in 0.2 N NaOH at 37°C for 10 minutes and incubated with 3 M Na-bisulfite at 50°C for 16 hours. DNA was then purified using the Wizard cleanup system (Promega, Madison, WI) and desulfonated with 0.3 N NaOH at 25°C for 5 minutes. DNA was then precipitated with ammonium acetate and ethanol, washed with 70% ethanol, dried, and resuspended in H₂O.

We used a LINE1 repetitive element bisulfite/pyrosequencing assay to estimate global methylation.¹⁸ After bisulfite treatment, a 50 μ L polymerase chain reaction (PCR) was carried out in 60 mmol/L Tris-HCl pH = 8.8, 15mM ammonium sulfate, 0.5 mmol/L MgCl₂, 1mM dNTP mix, and 1 unit of Taq polymerase. The PCR primers used were: 10 pmol of 5'-TTTTTTGAGTTAGGTGTGGG-3', 1pmol of 5'-GGGACACCGCTGATCGTTTATCTCACTAAAAATACCAAACAA-3', and 10 pmol of a universal biotinylated primer 5'-GGGACACCGCTGATCGTTTA-3'. PCR cycling conditions were 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds for 35 cycles. The PCR product was purified and methylation quantitated using the PSQ HS 96 Pyrosequencing System (Pyrosequencing Inc, Westborough, MA). The sequencing primer for pyrosequencing was 5'-GGGTGGGAGTGAT-3'.

We also used bisulfite-pyrosequencing¹⁹ to analyze methylation of the *p15* gene. A 50- μ L PCR was carried out in 60 mmol/L Tris-HCl pH = 8.8, 15mM ammonium sulfate, 2 mmol/L MgCl₂, 1mM dNTP mix, and 1 unit of Taq polymerase. PCR primers used were 10 pmol of 5'-GTTTTTTTTTAGAAGTAATTTA-3', 1 pmol of 5'-GGACACCGCTGATCGTTTATCCTTCTACGACTTAAACC-3', and 10 pmol of a universal biotinylated primer 5'-GGGACACCGCTGATCGTTTA-3'. PCR cycling conditions were melting temperature of 95°C for 30 seconds, annealing for 45 seconds at a temperature of 50°C for three cycles, 48°C for four cycles, 46°C for four cycles, 44°C for four cycles, 42°C for 43 cycles, and extension temperature of 72°C for 45 seconds. The sequencing primer for pyrosequencing was 5'-TTTTTAGAAGTAATTTAGG-3'.

Statistics

Simple descriptive statistics were used in the analysis of the clinical efficacy of decitabine. Survival was computed using Kaplan-Meier curves and the provided *P* values are two-sided. A *P* value of .05 was considered significant. The methylation data generated was expressed as a percentage and showed a normal distribution. The data was summarized (mean, median, standard error of the mean) using the Excel software (Microsoft, Redmond, WA). *T* tests or paired *t* tests were used to compare methylation at different time points or in different groups, as appropriate. Provided *P* values are two-sided. A *P* value of .05 was considered significant.

RESULTS

Patients Studied

A total of 35 patients were registered on this study. Their clinical characteristics and diagnoses are summa-

rized in Table 1. All but four patients had evidence of imatinib resistance. The patients with imatinib intolerance had shown severe, repeated elevation in liver enzymes or rashes following treatment with that agent. Among the patients with resistance to imatinib, 30 showed hematologic and cytogenetic resistance while one showed cytogenetic resistance only. Twelve patients were on imatinib the week before starting therapy, while the remaining had interrupted therapy because of toxicity or lack of response for a median of 50.5 weeks (range, 1 to 100 weeks) before starting decitabine.

Response to Treatment

Previous studies have repeatedly shown that decitabine works slowly, and that multiple cycles are required for optimal response.^{5,15} For this reason, the protocol recommended that patients complete at least two cycles of therapy, regardless of response. Thirty of the 35 patients received at least two cycles of therapy. In five patients, only one cycle was administered because of disease progression (two patients), early death (one patient) and non-compliance (two patients).

Responses are summarized in Table 2. Complete hematologic responses were seen in 50%, 29%, and 17% of patients in CP, AP, and BP, respectively. The corresponding total hematologic response rate (complete response + partial response) was 83%, 41%, and 34%. Twenty patients required the use of hydroxyurea during their first two cycles. The use of hydroxyurea had no impact on responses. In responding patients, 17 achieved their best response after one cycle, three after two cycles, and two after > two cycles. Response duration had a median of 3.5 months (range, 2 to 13.5 months). Cytogenetic responses are also summarized in Table 2. Major cytogenetic responses were seen in 25%, 12%, and 17% of patients in CP, AP, and BP, respectively, while minor responses were

Table 1. Patient Characteristics

Characteristic	No. of Patients	%
Age, years		
Median	61	
Range	31-79	
Sex, male	18	51
CML phase		
Chronic	12	34
Accelerated	17	49
Blastic	6	17
Imatinib status		
Intolerance	4	11
Cytogenetic resistance only	1	3
Hematologic and cytogenetic resistance	30	86
Karyotype		
Philadelphia chromosome only	19	54
Additional cytogenetic changes	16	46
Prior therapy (other than hydroxyurea)		
Imatinib only	9	26
Imatinib and other drugs	26	74

Abbreviation: CML, chronic myelogenous leukemia

Table 2. Summary of Responses

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Hematologic Response										Cytogenetic Response					
CML Phase	N	CHR		PHR		HI		Total		Major		Minor		Total	
		No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Chronic	12	6	50	4	33	0	0	10	83	3	25	4	33	7	58
Accelerated	17	5	29	2	12	3	18	10	59	2	12	5	29	7	41
Blastic	6	1	17	1	17	1	17	3	50	1	17	1	17	2	33

Abbreviations: CML, chronic myelogenous leukemia; CHR, complete hematologic response; PHR, partial hematologic response; HI, hematologic improvement.

observed in 33%, 41%, and 33% of patients. Overall, evidence of some cytogenetic response was seen in 58%, 41%, and 33% of patients in CP, AP, and BP, respectively.

While some of the responses were of short duration, there were responses that lasted a year or more. To explore the clinical significance of achieving a response in this setting, we used Kaplan-Meier curves to plot the survival of patients with or without response to decitabine. Overall, patients in BP had a median survival of 4 months, patients in AP had a median survival of 12 months, and the median survival of patients in CP has not been reached yet. Because the majority of patients with CP responded and had a relatively favorable prognosis, we excluded them from analysis. There were 23 patients with CML AP or BP enrolled in the study, with 13 responders and 10 nonresponders. As shown in Figure 1, the survival of responders to decitabine was distinctly superior to that of nonresponders ($P = .03$). Of the 10 patients (seven AP, three BP) who did not respond, all died except one patient in AP who was subsequently treated with a novel tyrosine kinase inhibitor. Of the 13 patients (10 AP, three BP) who responded, seven are alive, thus accounting for the plateau observed in the survival curve. One patient in BP received a stem-cell transplant after responding to decitabine, and

remains in remission. The other six patients (all AP) received decitabine for a median of 6 months (range, 3 to 12 months) and subsequently relapsed. Three have received salvage therapy with novel tyrosine kinase inhibitors and three were treated with salvage chemotherapy.

Toxicity

A summary of toxicities is shown in Table 3. In general, non-myelosuppression-related toxicity was rare, with no grade 4 adverse effects. Eighteen patients had neutropenic fever while on study, and 28 (23%) of 124 of the total cycles were complicated by neutropenic fever. No patient died of neutropenia and sepsis following decitabine therapy, while two died of hemorrhage in the setting of severe thrombocytopenia while on therapy (one with BP and pre-existing thrombocytopenia and one in CP). Hematologic toxicities are difficult to assess in this population because of bone marrow compromise by leukemia. For example, 13 patients were thrombocytopenic at study entry. To address this issue more precisely, we analyzed a total of 64 courses of therapy that were administered to patients who were in CHR. In those 64 courses, nadir neutrophil count was $0.37 \times 10^9/L$ (range, 0.0 to 4.35), attained at a median of 33.5 days after therapy. Nadir platelet count was $94.5 \times 10^9/L$ (range, 2 to 548), attained at a median of 22 days after therapy.

DNA Methylation Analysis

We used a bisulfite/pyrosequencing assay to measure DNA methylation across LINE1 repetitive elements in

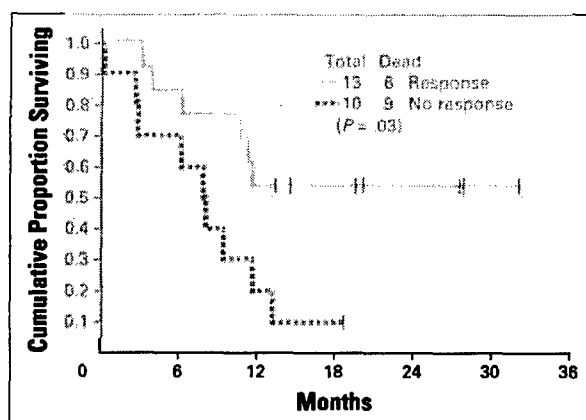


Fig 1. Kaplan-Meier curve of survival in chronic myelogenous leukemia accelerated phase or blastic phase by response to decitabine. Straight line: responders; dashed line: nonresponders.

Table 3. Grade 2 to 4 Toxicities Observed

Toxicity	Grade 2	Grade 3	Grade 4
Infection	NA	10	1
Hemorrhage	2	2	2
Pulmonary (dyspnea)	3	1	0
Cardiac	0	2	0
Mucositis	3	4	0
Nausea and vomiting	4	0	0
Diarrhea	2	0	0
Edema	5	0	0
Fatigue	5	0	0

Abbreviation: NA, not assessable.

Decitabine in CML

peripheral-blood mononuclear cells as a surrogate marker for global methylation. We first evaluated the reproducibility of the assay; 105 samples with values ranging from 32.1% to 81.3% were assayed in duplicate. The correlation between duplicates was $R = 0.82$ ($P < .001$). We next evaluated the stability of LINE1 methylation in CML over time. Fourteen patients had two separate samples independent of decitabine therapy assayed for LINE1 methylation at intervals ranging from 1 day to 2 years. The mean of sample 1 was 71.1%, and the sample 2 mean was 71.0% ($P = .9$ by paired t test for a difference). The correlation between the two independent samples from the same patient at different times (unrelated to decitabine) was $R = 0.84$ ($P < .001$).

We used this LINE1 assay to study global methylation in the patients entered onto this trial. Pretreatment samples were available on 30 patients. LINE1 methylation had a mean $\pm$ standard error of the mean of $71.4\% \pm 1.1\%$ (range, 56.5% to 85.8%). There was no difference in methylation between patients in CP ($72.1\% \pm 1.7\%$), AP ($70.3\% \pm 1.3\%$), or BP ($74.4\% \pm 4.0\%$). On-treatment samples were available for 22 patients for the first cycle. Typically, samples were collected before or on the first day of treatment, at the end of week 1 of therapy (day 5), at the end of week 2 of therapy (day 12), and at recovery of counts (days 30 to 50). In these 22 patients, methylation decreased from $71.3\% \pm 1.4\%$ to $60.7\% \pm 1.4\%$ at day 5 ($P < .001$) and $50.9\% \pm 2.4\%$ at day 12 ($P < .001$ compared with day 5), increasing back to $66.5\% \pm 2.7\%$ ($P =$ not significant compared to pretreatment) at the time of recovery (Fig 2). Representative examples of methylation dynamics in individual patients are shown in Figure 3.

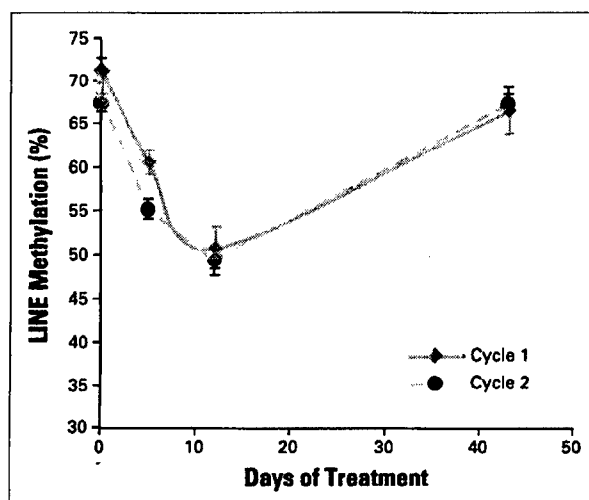


Fig 2. Methylation dynamics following cycle 1 (straight line) or cycle > 1 (dashed line) of decitabine therapy.

Most patients on study received more than one cycle, and samples were available for analysis from a total of 47 cycles administered to 22 patients (19 cycle 2, 12 cycle 3, 16 cycle > 3). This allowed us to determine the dynamics of hypomethylation after subsequent cycles. Overall, the decreases in methylation in cycles 2 and greater were similar to cycle 1. Methylation decreased from $67.4\% \pm 1.1\%$ pretreatment to $55.2\% \pm 1.1\%$ at day 5, $49.5\% \pm 1.7\%$ at day 12 and recovered to $67.4\% \pm 1.0\%$ at count recovery (Fig 2). The study allowed dose reduction for toxicity in subsequent cycles. Of the 47 cycles analyzed, 24 were given at full dose and 23 at a dose reduced by one third (10 mg/m^2 IV daily for 5 days per week on 2 consecutive weeks). Interestingly, the lower dose resulted in lower degrees of hypomethylation at days 5 ($57.0\% \pm 1.7\%$ v $53.4\% \pm 1.4\%$; $P = .1$) and at day 12 ($54.3\% \pm 2.4\%$ v $45.2\% \pm 2.1\%$; $P < .008$; Fig 4).

We analyzed the relationship between induction of hypomethylation during cycle 1 and response in CML. Of the 22 patients with samples available, 10 had partial or complete hematologic responses, while 12 had no responses. Figure 5 shows methylation dynamics in responders versus nonresponders. LINE1 methylation at baseline and at the end of week 1 did not correlate with subsequent responses. However, the degree of LINE1 hypomethylation at the end of therapy (day 12) was paradoxically higher in patients who did not subsequently respond to therapy. Thus, the absolute decrease in methylation was $14.5\% \pm 3.0\%$ versus $26.8\% \pm 2.7\%$ in responders versus nonresponders ($P = .007$) and the relative decrease in methylation was $20.4\% \pm 4.3\%$ versus $36.4\% \pm 3.7\%$ in responders versus nonresponders ($P = .012$).

One hypothesis to explain the paradoxical correlation between more profound hypomethylation and a lack of response to decitabine is a cell-death mechanism of response, whereby the most hypomethylated cells die rapidly in patients sensitive to therapy, while cells resistant to therapy can withstand higher degrees of hypomethylation without cell death. If this hypothesis were correct, by extension, resistance to decitabine would be distal to hypomethylation induction (ie, nonpharmacologic). This hypothesis can be tested by examining the relationship between hypomethylation induction and maintenance of response or relapse during therapy. Of the 47 cycles administered subsequent to cycle 1, for which samples are available, 19 were associated with maintained or improved response, 17 with consistent lack of response, and 11 with a relapse on therapy (ie, response in the previous cycle, loss of response and progression during the current cycle). We therefore examined hypomethylation induction in these three situations. In the cycles where remissions were maintained, the relative decrease in methylation was $10.9\% \pm 3.3\%$ at day 5 and $18.6\% \pm 4.8\%$ at day 12, compared with nonresponders at $22.8\% \pm 2.6\%$ at day 5 ($P = .008$) and

Issa et al

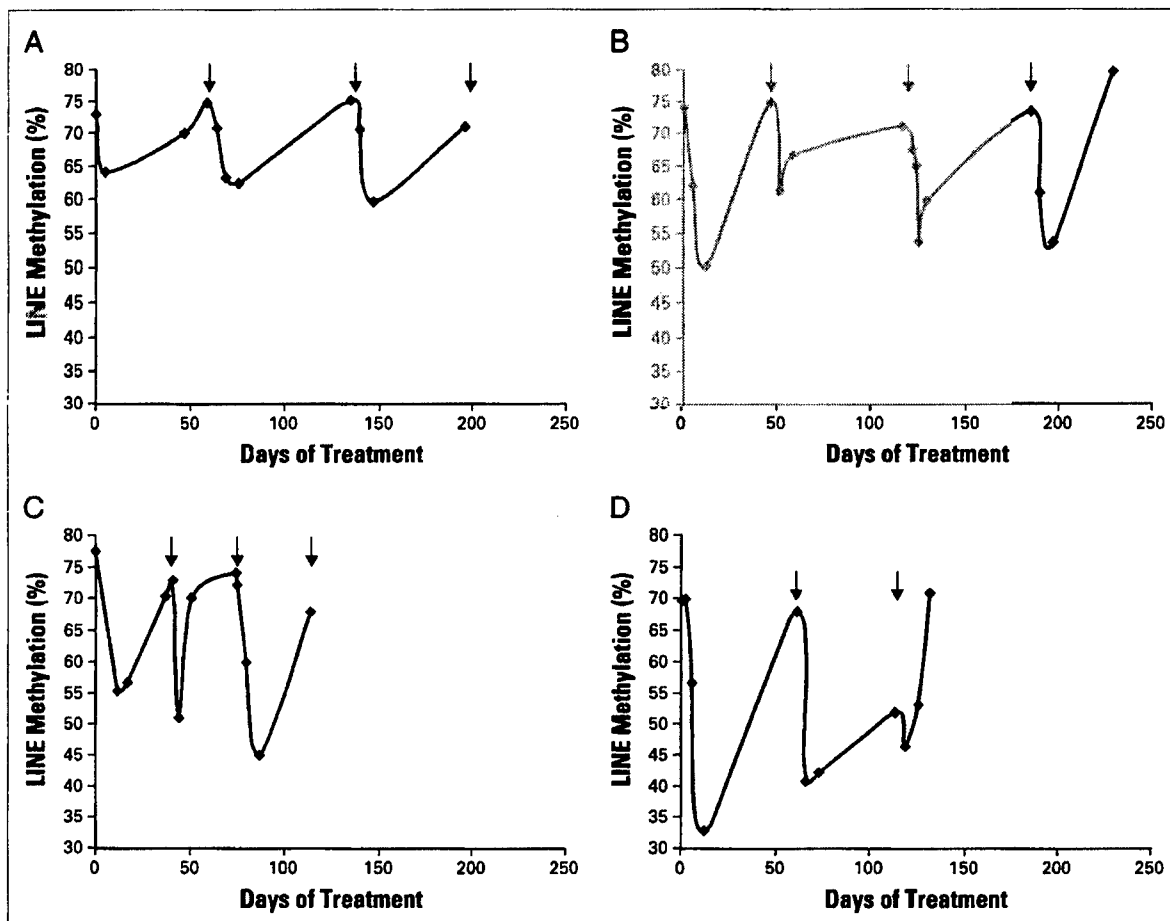


Fig 3. Methylation dynamics in four individual patients (A, B, C, D). Patients were started on treatment on day 1, and subsequent cycles are indicated by arrows.

30.7% $\pm$ 3.9% at day 12 ($P = .06$), respectively. Remarkably, in patients who responded and subsequently lost their response while on therapy, the relative decrease in methylation was intermediate, with a decrease of $17.5\% \pm 1.9\%$ at day 5 and $25.8\% \pm 5.6\%$ at day 12. In this latter group, the decreases at days 5 and 12 in the relapsing cycle were all the more remarkable when one compares them to the decreases seen during cycle 1 (for the same patients): $11.9\% \pm 3.4\%$ at day 5 and $25.1\% \pm 5.0\%$ at day 12. Thus, hypomethylation induction was similar or higher in relapse cycles compared to initial cycles.

Finally, we studied interactions between global methylation or methylation of the $p15^{INK4b}$ gene and response in these patients. As measured by LINE1, there was no correlation between global methylation and response; thus, LINE1 methylation had a mean of 69.3% in responders compared to 73.2% in nonresponders ($P = .1$). $p15^{INK4b}$ was densely methylated (methylation density $> 10\%$) in only three patients, precluding meaningful correlations with response. Nevertheless, of these three patients, one in BP was nonassessable, one in AP did not

respond, and another patient in AP had a transient, minimal response (hematologic improvement).

DISCUSSION

In this phase II study, we demonstrate unequivocal single-agent activity for decitabine in patients with CML in all phases who were refractory to, or intolerant of, imatinib. Toxicities were generally mild, and limited to myelosuppression. The response rates observed were broadly similar (or possibly better) than what was previously observed for single-agent decitabine at a higher dose in patients with CML before the availability of imatinib.¹⁵ Thus, the in vivo results are consistent with in vitro data suggesting lack of cross resistance between imatinib and decitabine.²⁰ Indeed, the mechanisms of action of these two agents are very distinct, and resistance to imatinib is mediated by mutations in *BCR/ABL* in many cases, mutations that would not be expected to alter sensitivity to decitabine.

The responses observed were generally short lived, although long-lasting responses were occasionally seen.

Decitabine in CML

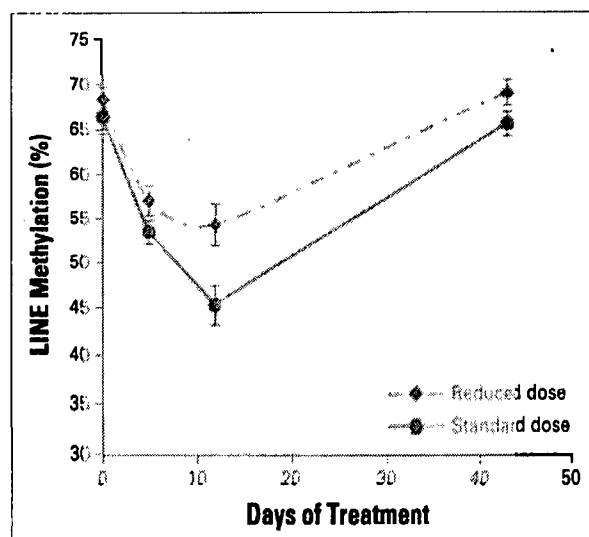


Fig 4. Methylation dynamics for cycles > 1 given at standard dose or reduced dose (reduced by one third).

Nevertheless, they appeared to be of clinical benefit, particularly in AP and BP, and decitabine may be a good choice as a bridge to stem-cell transplantation or for palliative care in these patients. The prognosis of patients with CML who develop resistance to imatinib is generally poor,¹³ and novel therapies are needed in this situation. Ultimately, the activity of decitabine could be boosted by combining it with other epigenetic acting agents such as histone deacetylase inhibitors,²¹ with retinoic acid or other biologic agents that exploit the epigenetic reactivation of gene expression,² or with apoptosis inducers to exploit the lowering of the apoptotic threshold induced by this drug.²² It would also be of interest to combine decitabine with imatinib, particularly in advanced stages of CML, and there is *in vitro* evidence of synergy between these two agents.²⁰

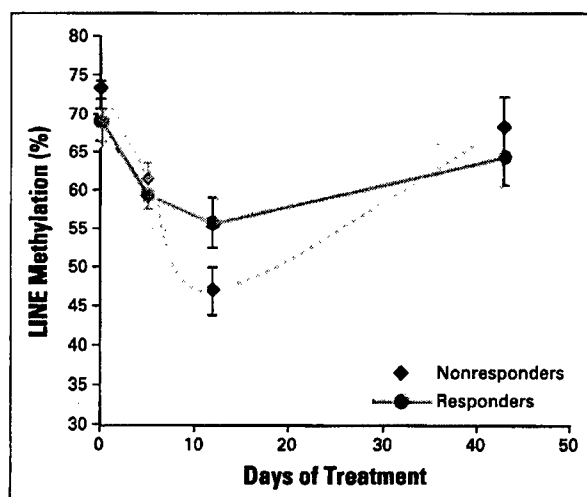


Fig 5. LINE1 methylation dynamics after cycle 1 of decitabine in patients who subsequently responded (straight line) or did not respond (dashed line) to therapy.

tabine with imatinib, particularly in advanced stages of CML, and there is *in vitro* evidence of synergy between these two agents.²⁰

The mechanisms of response to decitabine are of considerable interest. *In vivo* hypomethylation following treatment with this agent has previously been observed.^{6,23} In a study performed across multiple doses, it appeared that induction of global hypomethylation within 5 days of therapy was required to achieve response to this agent (Yang et al, manuscript submitted for publication). Nevertheless, the events downstream of hypomethylation are unknown; does the drug result in clinical responses through neoplastic cell death, differentiation, senescence, or via other mechanisms, such as an immune response or antiangiogenesis? Here, we addressed this issue further in a proliferative disease (CML) in patients treated with a uniform dose (at least for the first cycle). At day 5 after therapy, induction of hypomethylation could be observed in nearly all patients, and there was no difference between responders and nonresponders. WBC counts were also largely unchanged. Remarkably, at day 12, hypomethylation was less pronounced in responders than nonresponders, and WBC counts had started declining.

Several interpretations of the above findings are possible. A high degree of hypomethylation could actually be counterproductive in terms of clinical activity. This seems counterintuitive, but it remains possible that more hypomethylation reflects an effectively higher dose that could then suppress responses, perhaps via immunosuppression as argued previously.⁶ However, the lack of difference in methylation at day 5 favors alternate hypotheses. It is possible that, in responders, decitabine induces cell cycle arrest or differentiation, thus precluding further drug incorporation and proliferation and resulting in lower levels of methylation at day 12. Measuring differentiation markers and cell cycle parameters after decitabine therapy could help address this hypothesis. However, responses to decitabine are almost always preceded by decreases in peripheral-blood counts and clearance of bone marrow blasts, with no overt evidence of cellular differentiation, suggesting actual neoplastic cell death. An intriguing possibility then is that responses occur by hypomethylation induction of cell death after an average of 5 days. If this hypothesis were true, patients sensitive to decitabine would have more elimination of hypomethylated cells, and would appear to have paradoxically higher levels of methylation after the first wave of cell death. This should be confirmed by studies of apoptosis induction following decitabine therapy *in vivo*. It is also important to note that (1) effects on LINE methylation may be different than effects on gene-specific methylation; thus, no conclusion can be drawn regarding the contribution of gene-specific hypomethylation to responses, and (2) all the molecular studies reported here were based on cells obtained from

the peripheral blood rather than bone marrow, and that effects on bone marrow cells (or leukemia stem cells) could be different.

Mechanisms of primary or secondary *in vivo* resistance to decitabine are unknown. In particular, it is not clear whether resistance is pharmacologic (ie, drug incorporation and induction of hypomethylation) or downstream of hypomethylation induction, such as by upregulation of apoptosis inhibitors. The cell-death hypothesis proposed earlier predicts that, in some cases, resistance to decitabine would be nonpharmacologic. In fact, when responding patients develop resistance to this agent while on therapy, the cycle associated with resistance was found to induce a similar or higher degree of hypomethylation than previous cycles, which is consistent with the hypothesis of a nonpharmacologic mechanism of resistance. These clinical observations raise a number of biologic questions that should be tested further.

The recovery of methylation following decitabine therapy is of interest. Here, we show that recovery occurs universally by day 30 to 50 after therapy, and coincides with recovery from myelosuppression. Two factors contribute to this recovery. First, gradual elimination of the cells with the highest degrees of hypomethylation leaves behind cells that did not hypomethylate, thus giving the illusion of remethylation while one is actually observing clonal replacement. In parallel, it is clear that once decitabine is no longer present, DNA methyltransferase activity recovers by new protein synthesis and thus remethylates the genome. The stimuli for this remethylation are unknown. Given that hypomethylation by decitabine is only partial, a plausible hypothesis is that residual regional

methylation serves as a nidus for the observed remethylation. It is also worth noting that no cumulative hypomethylation was observed, which is reassuring vis-à-vis long-term safety of this therapy. The issue of safety and carcinogenic potential of hypomethylation therapy, however, needs to be addressed in clinical studies with longer follow-up.

We conclude that decitabine has single-agent activity in patients with CML refractory or intolerant to imatinib therapy. The dynamics of hypomethylation in reference to responses suggest that decitabine induces remission via hypomethylation-mediated clearing of neoplastic cells, and indicate that resistance to this agent in CML is nonpharmacologic and possibly due to disturbances in apoptotic signals.

Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Consultant: Jean-Pierre Issa, SuperGen, MGI Pharma. Guillermo Garcia-Manero, SuperGen, MGI Pharma. Research Funding: Jean-Pierre Issa, SuperGen; Guillermo Garcia-Manero, SuperGen; Hagop M. Kantarjian, MGI Pharma, SuperGen. For a detailed description of these categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section of Information for Contributors found in the front of every issue.

REFERENCES

- Santini V, Kantarjian HM, Issa JP: Changes in DNA methylation in neoplasia: Pathophysiology and therapeutic implications. *Ann Intern Med* 134:573-586, 2001
- Egger G, Liang G, Aparicio A, et al: Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 429:457-463, 2004
- Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 349:2042-2054, 2003
- Juttermann R, Li E, Jaenisch R: Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci U S A* 91:11797-11801, 1994
- Leone G, Voso MT, Teofili L, et al: Inhibitors of DNA methylation in the treatment of hematological malignancies and MDS. *Clin Immunol* 109:89-102, 2003
- Issa JP, Garcia-Manero G, Giles FJ, et al: Phase I study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (Decitabine) in hematopoietic malignancies. *Blood* 103:1635-1640, 2004
- Druker BJ: Perspectives on the development of a molecularly targeted agent. *Cancer Cell* 1:31-36, 2002
- Sawyers CL, Hochhaus A, Feldman E, et al: Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: Results of a phase II study. *Blood* 99:3530-3539, 2002
- Talpaz M, Silver RT, Druker BJ, et al: Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: Results of a phase 2 study. *Blood* 99:1928-1937, 2002
- Kantarjian H, Sawyers C, Hochhaus A, et al: Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* 346:645-652, 2002
- Gorre ME, Sawyers CL: Molecular mechanisms of resistance to STI571 in chronic myeloid leukemia. *Curr Opin Hematol* 9:303-307, 2002
- Hochhaus A, Hughes T: Clinical resistance to imatinib: Mechanisms and implications. *Hematol Oncol Clin North Am* 18:641-66, ix, 2004
- Branford S, Rudzki Z, Walsh S, et al: Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 102:276-283, 2003
- Shah N, Nicoll J, Nagar B, et al: Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2:117-125, 2002
- Kantarjian HM, O'Brien S, Cortes J, et al: Results of decitabine (5-aza-2'-deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer* 98:522-528, 2003
- Kantarjian HM, Cortes J, O'Brien S, et al: Imatinib mesylate (STI571) therapy for Philadelphia chromosome-positive chronic myelogenous leukemia in blast phase. *Blood* 99:3547-3553, 2002

Decitabine in CML

17. Clark SJ, Harrison J, Paul CL, et al: High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 22:2990-2997, 1994

18. Yang AS, Estecio MR, Doshi K, et al: A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 32:e38, 2004

19. Colella S, Shen L, Baggerly KA, et al: Sensitive and quantitative universal Pyrosequencing

methylation analysis of CpG sites. *Biotechniques* 35:146-150, 2003

20. La Rosee P, Johnson K, Corbin AS, et al: In vitro efficacy of combined treatment depends on the underlying mechanism of resistance in imatinib-resistant Bcr-Abl-positive cell lines. *Blood* 103:208-215, 2004

21. Cameron EE, Bachman KE, Myohanen S, et al: Synergy of demethylation and histone deac-

etylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 21:103-107, 1999

22. Jones PA: Cancer, Death and methylation. *Nature* 409:141, 143-144, 2001

23. Daskalakis M, Nguyen TT, Nguyen C, et al: Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-aza-2'-deoxycytidine (decitabine) treatment. *Blood* 100:2957-2964, 2002