Phase I Study of Cloretazine (VNP40101M), a Novel Sulfonylhydrazine Alkylating Agent, Combined with Cytarabine in Patients with Refractory Leukemia

Francis Giles,[1] Srdan Verstovsek,[1] Deborah Thomas,[1] Stanton Gerson,[2] Jorge Cortes,[1] Stefan Faderl,[1] Alessandra Ferrajoli,[1] Farhad Ravandi,[1] Steven Kornblau,[1] Guillermo Garcia-Manero,[1] Elias Jabbour,[1] Susan O'Brien,[1] Verena Karsten,[3] Ann Cahill,[3] Karen Yee,[1] Maher Albitar,[1] Mario Sznol,[3] and Hagop Kantarjian[1]

Abstract **Purpose:** Cloretazine (VNP40101M) is a novel sulfonylhydrazine alkylating agent with significant antileukemia activity. A phase I study of cloretazine combined with cytarabine (1-β-D-arabinofuranosylcytosine, ara-C) was conducted in patients with refractory disease.

Design: Ara-C was given i.v. at a fixed dose of 1.5 gm/m^2/d by continuous infusion for 4 days (patients ages <65 years at time of diagnosis) or 3 days (patients ages ≥65 years). Cloretazine was given i.v. over 15 to 60 minutes on day 2 at a starting dose of 200 mg/m^2, with escalation in 100 mg/m^2 increments in cohorts of three to six patients until a maximum tolerated dose was established. The DNA repair enzyme O^6-alkylguanine DNA alkyltransferase (AGT) was measured at baseline.

Results: Forty patients, including 32 with acute myeloid leukemia, received 47 courses of treatment. Complete responses were seen at cloretazine dose levels of ≥400 mg/m^2 in 10 of 37 (27%) evaluable patients, and in this patient subset, AGT activity was significantly lower in patients that responded to treatment than in patients who did not ($P \leq 0.027$). Dose-limiting toxicities (gastrointestinal and myelosuppression) were seen with 500 and 600 mg/m^2 of cloretazine combined with the 4-day ara-C schedule but not seen with the 3-day schedule.

Conclusion: The recommended cloretazine dose schedule for future studies is 600 mg/m^2 combined with 1.5 gm/m^2/d continuous infusion of ara-C for 3 days. The cloretazine and ara-C regimen has significant antileukemic activity. AGT activity may be a predictor of response to cloretazine.

Patients with relapsed or refractory leukemia have a poor prognosis, and more effective agents are needed to improve complete remission (CR) rates and durations of response (1, 2). Many novel compounds with unique and targeted mechanisms of action are being introduced into the clinic. Current data suggest that most will require combination with standard cytotoxic agents in therapeutic antileukemia regimens (3–7). The development of more active, better tolerated cytotoxic agents continues to be important in attempting to improve therapy for patients with leukemia.

DNA alkylating agents are an important class of antileukemia agents, sharing the ability to damage DNA and/or impair its replication (5, 7, 8). Among the class, there is a broad spectrum of antitumor activity and toxicity, attributable to individual biological properties of the agents, including the type of DNA damage, relative specificity of attacking DNA versus other cellular components, the DNA repair mechanisms used by the cell in response to the agent, uptake and distribution of the agent in malignant and normal cells, and the relative susceptibility of the agent to leukemia resistance mechanisms (9–12). With such diverse mechanisms capable of affecting efficacy and/or tolerability, there is an ongoing search for novel alkylating agents with potential advantages over currently available drugs. This search is particularly important in patients with hematologic malignancies, where alkylating agents are core components of many standard regimens.

In preclinical studies, Cloretazine (VNP40101M; Fig. 1), a novel sulfonylhydrazine alkylating agent, showed potential advantages over some standard alkylating agents (13–17). Cloretazine first undergoes activation to yield 90CE [1,2-bis(methylsulfonyl)-1-(2-chloroethyl) hydrazine] and methylisocyanate. The 90CE rapidly produces an alkylating, chloroethylating species, similar to the chloroethylating

Authors' Affiliations: [1]Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; [2]Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, Ohio; and [3]Vion Pharmaceuticals, Inc., New Haven, Connecticut

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Requests for reprints: Francis J. Giles, Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, 1400 Holcombe Boulevard, Box 428, Houston, TX 77030. Phone: 713-792-7305; Fax: 713-794-4297; E-mail: frankgiles@aol.com.

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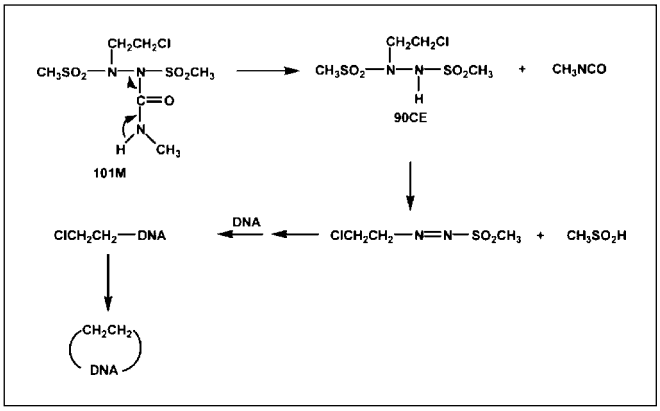

Fig. 1. Cloretazine activation.

species generated by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine; refs. 17, 18). Unlike BCNU, cloretazine does not generate hydroxyethylating, vinylating, or aminoethylating species, and alkylation is relatively specific to the O^6 position of guanine (18–20). As a consequence of the different alkylating and isocyanate species generated, *in vitro*, cloretazine produces more cross-links and fewer DNA single-strand nicks than BCNU (18).

Cloretazine has broad spectrum antitumor activity in animal models and crosses the blood-brain barrier (17, 21). It has significant activity against cell lines derived from both solid tumors and hematologic malignancies, including leukemia cell lines selected for resistance to other alkylating agents. Doses of ≥20 mg/kg (60 mg/m^2) administered i.p. produced 100% long-term survival in 1-day-old i.p. implanted L1210 leukemia and P388 leukemia (17). Cloretazine was associated with long-term survival in mice implanted with L1210 cell lines selected for resistance to BCNU, cyclophosphamide, or melphalan. The cloretazine doses that were effective in sensitive and resistant L1210-bearing mice (20-60 mg/kg or 60-180 mg/m^2) were modestly myelosuppressive when administered to non–tumor-bearing mice.

Based on these preclinical data, the first phase I study of cloretazine was initiated in patients with solid tumors using a schedule of short i.v. infusion every 4 to 6 weeks (22). Thrombocytopenia was the dose-limiting toxicity (DLT), and the maximum tolerated dose (MTD) was 305 mg/m^2 with platelet and neutrophil nadirs occurring at a median of 27 and 34 days, respectively. Of note was the lack of significant nonhematologic toxicity, suggesting that substantial dose escalation might be possible in patients with hematologic diseases. Thus, a phase I study of cloretazine was conducted in patients with refractory leukemia (23). Thirty-eight patients, including 28 with acute myeloid leukemia (AML) and five with myelodysplastic syndromes, received 52 courses of treatment. Non–dose-limiting, reversible infusion-related toxicities were the most frequent adverse event, occurring in 24 (63%) patients on the first course. Dose escalation was terminated at 708 mg/m^2 for prolonged myelosuppression in one of seven patients, and 600 mg/m^2 was selected as the recommended phase II study dose, with no significant extramedullary toxicity at this dose level. Two patients, one with myelodysplastic syndrome treated with 300 mg/m^2 and one with AML treated with 600 mg/m^2, achieved CR. As cloretazine has significant antileukemic activity and minimal extramedullary toxicity in patients with refractory disease, and cytarabine (1-β-D-arabinofuranosylcytosine, ara-C) is a standard active antileukemia drug (1), a phase I study of the combination of cloretazine and ara-C was conducted in patients with refractory leukemia.

For future studies of cloretazine, knowledge of key mechanisms of resistance may be important in developing approaches to select patients and improve antitumor activity. Evidence that initial resistance is mediated by the cellular enzyme O^6-alkylguanine DNA alkyltransferase (AGT), which removes initial monoadducts in DNA and prevents cross-linking, has been obtained both *in vitro* and *in vivo* preclinical studies (16, 24, 25). Thus, assessment of prestudy AGT expression was conducted in consenting study patients.

Patients and Methods

The study was reviewed and approved by the Institutional Review Board of the M.D. Anderson Cancer Center. All patients gave signed informed consent, indicating that they were aware of the investigational nature of this study.

Patient eligibility. Patients with relapsed or refractory leukemias, for which no standard therapy was anticipated to result in a durable remission, were eligible for study entry. Patients with untreated leukemia electing not to receive standard therapy were also eligible. Patients with active controlled infection, including chronic hepatitis, or with known central nervous system leukemia, were eligible. The protocol did not include age restrictions. Other eligibility criteria included the following: Eastern Cooperative Oncology Group performance score of ≤2; serum bilirubin of ≤1.5 mg/d; aspartate aminotransferase or alanine aminotransferase levels ≤3 times upper limit of normal; and serum creatinine of ≤2.0 mg/d. In the absence

Table 1. Baseline characteristics of 40 patients treated on study

Characteristics	*n* (%)
Diagnosis	
AML	32 (80)
Myelodysplastic syndromes	1 (3)
ALL	6 (15)
Chronic myeloid leukemia-blastic phase	1 (3)
Eastern Cooperative Oncology Group performance status	
0-1	37 (93)
2	3 (8)
Cytogenetics	
Diploid	11 (28)
Philadelphia chromosome [t(9:22)]	3 (8)
Miscellaneous	7 (18)
11q	1 (3)
+8	5 (13)
−5, −7	12 (30)
Insufficient metaphases	1 (3)
AML treatment status	
Untreated	4 (13)
First salvage	7 (22)
Second salvage	8 (25)
Third or more salvage	13 (41)

Table 2. Grade 3 or 4 toxicity by cloretazine dose level

Dose (mg/m^2)	No. patients	No. patients evaluable	Gastrointestinal	Hepatic	Other
200	6	4			
300	3	3			
400	6	6	1		
500	14	14	3	2	Fatigue (2)
600	11	10	2	1	Prolonged myelosuppression (1)

of rapidly progressive disease, the interval from prior treatment with myelosuppressive cytotoxic agents could not be <2 weeks, and patients requiring hydroxyurea for control of peripheral blood cell counts must have discontinued the hydroxyurea at least 48 hours before treatment on study. Patients, regardless of rate of disease progression, were allowed no cytotoxic therapy other than hydroxyurea in the 2 weeks preceding study drug administration. All patients of childbearing potential agreed to use adequate contraception for the duration of the study. Pregnant or nursing patients were excluded and any woman of childbearing potential required a negative pregnancy test within the week before study entry. Additional ineligibility criteria included myocardial infarction within the previous 3 months, symptomatic coronary artery disease, arrhythmias not controlled by medication, uncontrolled congestive heart failure, and concomitant standard or investigational antileukemia treatment while on study; in addition, because the formulation of cloretazine contains 30% ethanol, concurrent treatment with disulfiram (Antabuse) was not allowed.

Table 3. Toxicity by cloretazine dose (mg/m^2)

Dose level	Toxicity	Grade			
		1	2	3	4
200 mg, 6 patients	Nausea/vomiting	4			
(2 early deaths)	Gastrointestinal	2	1		
	Infusion related	1			
	Hepatic	1	3		
	Rash		1		
300 mg, 3 patients	Nausea/vomiting	1	1		
	Gastrointestinal	3			
	Hepatic		1		
400 mg, 6 patients	Nausea/vomiting	3	2	1	
	Gastrointestinal	6	3		
	Hepatic	7	1		
	Facial flushing	1			
	Rash		1		
	Dry skin	1			
500 mg, 14 patients	Nausea/vomiting	6	2		
	Gastrointestinal	4	3	2	1
	Fatigue			2	
	Infusion related	5			
	Rash	1			
	Hepatic	5	2	2	
	Dry skin	1			
	Hand/foot syndrome		1		
	Alopecia	3			
	Fever	1			
600 mg, 11 patients	Nausea/vomiting	4	2		
	Gastrointestinal	8	1	2	
	Hepatic	6	2	1	
	Prolonged myelosuppression				1
	Rash		2		
	Alopecia	1	1		

Treatment and study design. Cloretazine was supplied by Vion Pharmaceuticals, Inc. (New Haven, CT) as a clear, colorless, slightly viscous, sterile, nonaqueous solution for i.v. administration in 10-mL vials containing 100 mg of cloretazine, 3 mL of anhydrous ethyl alcohol, 7 mL of polyethylene glycol 300, and 6 mL of citric acid. Cloretazine was stored under refrigeration, at 2°C to 8°C (36-46°F), except when being prepared for injection. For administration to patients, cloretazine was diluted in 5% dextrose injection, USP up to concentrations of 4 mg/mL in a final volume of 50 to 500 mL. For total doses up to 800 mg, the final infusion volume was 250 mL administered over 15 to 30 minutes. For total doses of 801 to 1,600 mg, the final infusion volume was 500 mL administered over 30 to 60 minutes. Dilutions were prepared in glass or plastic containers not containing di-(ethylhexyl)phtalate.

Ara-C was given i.v. at a fixed dose of 1.5 g/m^2/d by continuous infusion on days 1 to 4 in patients ages <65 years at time of diagnosis or on days 1 to 3 in patients ages ≥65 years. Cloretazine was administered by i.v. infusion using a polyethylene-lined administration set inserted into a peripheral or central vein. Management of neutropenia, neutropenic fever, thrombocytopenia, mucositis, or diarrhea followed standard institutional guidelines. As transient infusion-related reactions (one or more of the following: facial flushing, headache, nausea, dizziness, and asymptomatic hypotension) were observed in the single-agent study at doses ≥400 mg/m^2 (23), patients on the current study were pretreated with antihistamines. For severe acute drug-related reactions, infusions were stopped and supportive care administered until the reaction resolved. Subsequently, the infusion could be restarted and completed at 50% of the original infusion rate. If a severe reaction recurred at the lower dose rate, no further treatment was given for that cycle.

Patients were evaluated on the day of therapy and at least thrice weekly and as clinically indicated while on protocol. At each evaluation, patients were assessed for toxicity, and CBC with platelets and differential, serum chemistries, and liver function tests were obtained. Patients were evaluated for response, including a bone marrow aspirate and biopsy ~4 weeks after each dose. Those without evidence of

Table 4. Response to cloretazine and ara-C regimen in 40 patients with refractory or high-risk leukemia

Response	*n* (%)
CR	4 (10)
CR_P	6 (15)
Resistant	24 (60)
Early death	3 (8)
Aplastic death	3 (8)

Table 5. Characteristics of responding patients

Patient study no.	11	12	32	16	29
Diagnosis	AML	AML	AML	AML	AML
Sex	Male	Female	Male	Female	Female
Age	51	51	70	24	56
Baseline cytogenetics	Diploid	Diploid	−5/−7	Diploid	−5/−7
Treatment status	Previously treated AML	Previously treated AML	Untreated AML	Previously treated AML	Untreated AML (previously treated ALL)
Duration 1st CR (wk)	0	0	NA	31	NA
Baseline					
WBC	0.6	15.7	1.7	58	3.8
ANC	462	0	493	6380	532
Hb	8.5	9.5	8.5	12.7	8.5
Platelets	68	11	36	100	42
Bone marrow blasts	22	97	24	67	64
Lowest					
WBC	0.1	0.1	0.1	0.2	0.1
Platelets	2	3	5	10	1
Response to cloretazine + ara-C	CR	CR_P	CR_P	CR	CR_P
Duration cloretazine + ara-C induced CR (wk)	64*	5	2†	58*	19
Total courses	2	1	1	1	1
Cloretazine + ara-C postremission therapy	None	None	None	DLI	None
Cloretazine (mg/m^2, day 2)/ara-C schedule (g/m^2) × d	400/1.5 × 4	400/1.5 × 4	500/1.5 × 3	500/1.5 × 4	500/1.5 × 4

Abbreviations: ANC, absolute neutrophil; Hb, hemoglobin.
*Continues in remission.
†Died in CR from sepsis.
‡Died after stem cell transplantation from cardiac failure.

disease progression or severe or life-threatening drug-related toxicity were eligible to receive additional cycles of treatment every 4 weeks for up to six cycles.

A minimum of three patients were entered at each dose level. No new patients were entered at an escalated dose level until all patients at the current dose level had been observed for a minimum of 3 weeks. If none of the first three patients at a dose level experienced first-cycle DLT, new patients were entered to the next higher dose level. If one of the first three patients in a dose level developed first-cycle DLT, up to three more patients were required to start treatment at that same dose level. When two or more patients experienced first-cycle DLT, no further patients could be treated at that dose level. The MTD was defined as the highest dose level in which less than two patients of six developed first-cycle DLT.

The starting dose of cloretazine was 200 mg/m^2 and was escalated by 100 mg/m^2 increments for successive cohorts. When regimen-related DLT was observed sufficient to conclude that a dose level was above the MTD, the next lower dose level was expanded to six patients as necessary, and if the MTD was not exceeded, an intermediate dose level between the dose above the MTD and the next lower dose level could be evaluated.

Patients developing severe and life-threatening toxicity in which additional treatment posed a substantial risk for toxicity had treatment discontinued permanently. Patients developing a DLT but continuing on the study would have a dose reduction to the next lower dose level in the next cycle. Dose reductions for individual patients were permanent, and all toxicity for the previous cycle had to have returned to grade ≤1 before beginning a new cycle of therapy.

Toxicity was graded on a scale of 0 to 5 using the National Cancer Institute Common Toxicity Criteria (version 3.0). All patients who received any therapy on study were considered evaluable for toxicity. DLT was defined as any grade 3 nonhematologic toxicity that does not resolve to grade ≤2 within 24 hours, any grade 4 nonhematologic toxicity, or myelosuppression manifested as bone marrow hypoplasia for ≥42 days with a bone marrow cellularity of ≤5% and no evidence of leukemia. Because certain DLTs (e.g., rapidly reversible grade 3 liver function test elevations) preclude dose escalation but are tolerable and manageable, with approval of the Institutional Review Board, accrual could continue to a dose level in which reversible and manageable DLTs were observed. This dose level could be defined as the MTD with the provision that all toxicities, including DLTs, were rapidly reversible and manageable.

Response criteria. CR in patients with AML, myelodysplastic syndromes, or acute lymphocytic leukemia (ALL) was defined as normalization of the blood and bone marrow with ≤5% blasts, a granulocyte count of $\geq 1 \times 10^9$/L, and a platelet count of $\geq 100 \times 10^9$/L. Patients who met these criteria in the peripheral blood but still had 6% to 25% marrow blasts were considered to have a partial remission as long as the blast count had decreased by at least 50% from pre-study treatment with cloretazine. CR with incomplete platelet recovery (CR_P) was defined as for CR, but with platelet counts remaining below 100×10^9/L. Other responses were considered as failures and categorized as (a) early death if death occurred within 2 weeks from start of therapy; (b) aplastic death if death occurred during therapy without evidence of hematologic recovery and with too few cells to count; (c) died with response unknown if death occurred without bone marrow or peripheral blood picture indicative of aplasia or progressive disease; (d) secondary resistance if treatment induced a transient reduction in marrow blast + promyelocytes to ≤10%, unless the blast percentage fell under this variable at the time of study entry; and (e) primary resistance if any marrow during the course did not

Table 5. Characteristics of responding patients (Cont'd)

15	25	31	27	38
AML	AML	CML BC	ALL	ALL
Female	Male	Female	Male	Male
53	66	44	49	55
−5/−7	Diploid	Philadelphia	Philadelphia	+8
Previously treated AML	Untreated AML	Previously treated with Gleevec for chronic and blastic phase	Previously treated ALL	Previously treated ALL
0	NA	NA	34	80
1.4	2.7	2.5	12	3.7
210	972	775	5520	3071
8.7	10.8	9.1	11.9	13.6
19	172	39	88	157
30	27	36	59	38
0.1	0.1	0.1	0.1	0.3
4	7	6	3	4
CR_P	CR	CR_P	CR_P	CR
39	6[†]	20[‡]	18	4
1	1	1	1	2
Ara-C; MUD (FAMP/Bu/ATG)	Low-dose ara-C	MUD transplant (Bu/Cy/ATG)	POMP + Gleevec/Hydrea	None
500/1.5 × 4	600/1.5 × 3	500/1.5 × 4	600/1.5 × 4	600/1.5 × 3

show too few cells to count or blasts + promyelocytes of ≤10%. For patients with blastic phase chronic myeloid leukemia, return to chronic phase was considered a CR. This was defined as <15% blasts in the bone marrow and peripheral blood, <30% blasts plus promyelocytes in bone marrow and peripheral blood, and <20% basophils. CR_P was defined as for CR, but with platelet counts remaining below 100 × 10^9/L. Extramedullary AML or blastic phase chronic myeloid leukemia was considered a partial remission if the measurable disease was reduced by ≥50% and a CR if there was complete resolution of all measurable disease.

Assay of O^6-alkylguanine DNA alkyltransferase activity in peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation over Ficoll-Paque (Amersham, Arlington Heights, IL) within a few hours of drawing. Freshly isolated peripheral blood mononuclear cells were stored at −70°C until assayed. Cell extracts were prepared by sonication and the DNA concentration was determined using Hoesch dye 33258. The activity of AGT was measured as removal of the [^{3}H]methyl adduct from the O^6 position of guanine in DNA by incubating cell extracts with [^{3}H]methyl DNA substrate that was prepared by reacting calf thymus DNA with [^{3}H]methylnitrosourea as previously described (26). Briefly, cell extract was incubated with [^{3}H]methyl DNA in cell extract buffer [containing 70 mmol/L HEPES (pH 7.8), 0.1 mmol/L EDTA, 5% glycerol, 1 mmol/L DTT, and 25 μmol/L spermidine] in the total volume of 300 μL for 60 minutes at 37 °C. The reaction mixture contained excess substrate DNA such that the total AGT activity could be determined. The reaction was stopped with 7.5% trichloroacetic acid at 4°C for 30 minutes. The precipitate was collected by centrifugation at 13,000 rpm for 2 minutes and washed with 300 μL of 80% ethanol. Methylated purines were liberated from precipitated DNA during hydrolysis with 150 μL of 0.1 NHCl at 80°C for 1 hour. [^{3}H]O^6-methylguanine and [^{3}H]N^7-methylguanine (which was constant during the incubation and served as internal standard) in supernatants were separated by reverse-phase high-performance liquid chromatography and quantitated by liquid scintillation counting.

Results

Patient characteristics. Forty patients were enrolled on study between July 2003 and May 2004. Baseline characteristics are summarized in Table 1. Twenty (50%) patients were male. The median patient age was 54 years (range, 22-76), and performance status was 0 or 1 in 37 patients (93%). Thirty-two (80%) patients had AML by the WHO criteria (27). One AML patient had extramedullary disease without evidence of bone marrow relapse. One (3%) patient had myelodysplastic syndrome (chronic myelomonocytic leukemia). Of the 33 patients with AML or myelodysplastic syndrome, five received cloretazine and ara-C as induction therapy. Of these patients, who were considered unfit for standard AML induction chemotherapy because of severe comorbid medical conditions, two had received no prior antileukemia therapy, one targeted therapy with PKC512 (28), another with homoharringtonine (29).

One patient had been previously treated for ALL with hyperfractionated CVAD plus Rituximab. This patient achieved a CR of 2-year duration, before representing with AML. Seven patients with AML received cloretazine plus ara-C as a first salvage attempt, one with a first CR duration of 8 months, two with a first CR duration of <6 months, and four with primary refractory disease. Eight patients with AML received cloretazine and ara-C as a second salvage attempt, 13 as a third or subsequent salvage. Six (15%) patients had ALL; and one (3%) patient had chronic myeloid leukemia in blastic phase. Twenty-five (63%) patients had unfavorable cytogenetics (1 with an 11Q abnormality, 5 with trisomy 8, 12 with −5 and/or −7 abnormalities, and 7 multiple chromosomal abnormalities); 11 (28%) patients had diploid cytogenetics; three (8%) had the Philadelphia chromosome (including two with ALL); and one (3%) patient had insufficient metaphases

for chromosomal analysis. On the first course of therapy, 13 (33%) patients received the 3-day ara-C schedule, 26 (66%) received the 4-day schedule, and one patient died after receiving <24 hours of ara-C, and thus did not receive cloretazine before his death. Seven patients received second courses of therapy: four with same doses as in the first course and three received a reduction in either agent.

Toxicity. Thirty-seven patients were fully evaluable for toxicity; three patients died from rapidly progressive disease within the first 7 days of receiving therapy and were partially evaluable for toxicity. Forty patients received a total of 47 courses of treatment (range, 1-2; median, 1). Of 30 patients who did not respond, 11 had a normal absolute neutrophil or grade 1 and/or 2 neutropenia at baseline. Five of these patients developed grade 3 and/or 4 neutropenia, which resolved at a mean of 24 days (range, 18-35). The other six patients did not recover their absolute neutrophil. Recombinant growth factors were not administered. All patients received the treatment initially as an inpatient and 24 patients were subsequently readmitted for sepsis and/or disease progression. Gastrointestinal toxicities (including diarrhea, mucositis, colitis, anorexia, xerostomia, and dysgeusia) were the most common adverse events, with 36 occurrences in the first course. One patient at the 400 mg dose level had grade 3 nausea and vomiting which was transient, three patients each at the 500 mg dose level had transient anorexia, colitis, and diarrhea, respectively. Two patients at the 600 mg dose level had transient grade 3 colitis. Twenty-six episodes of nausea and vomiting, assessed separately from the other gastrointestinal toxicities, were observed during the first course of therapy. There were 31 episodes of hepatic toxicity. One patient treated at the 500 mg cloretazine dose level had grade 3 elevations in aspartate aminotransferase and alanine aminotransferase, both of which resolved within 3 days. A second patient treated at this dose level had grade 3 hyperbilirubinemia, which resolved within 2 days. A third patient at the 600 mg dose level developed transient grade 3 hyperbilirubinemia. Other toxicities included infusion-related events, fatigue, rash, dry skin, hand-foot syndrome, anorexia, facial flushing, prolonged myelosuppression, alopecia, and fever. No patient received prolonged infusions because of infusion reactions and no patient had the infusion discontinued for persistent infusion reactions. Grade 3 and 4 regimen-related adverse events occurring in course one are summarized in Table 2. For regimen-related toxicity occurring in course one, all grades are summarized in Table 3. DLTs were colitis (occurring in two patients at the 600 mg/m^2 and one patient at the 500 mg/m^2 dose levels) and prolonged myelosuppression (occurring at the 600 mg/m^2 dose level). All DLTs occurred with the 4-day ara-C dosing schedule. This led to an expansion of the 600 mg/m^2 cloretazine with the 3-day ara-C infusion cohort to include a further three patients. This schedule was not associated with DLT.

Response. Three patients died early and were not evaluable for response, one on day 2, before cloretazine infusion, another on day 6, the third on day 7. Overall responses are shown in Table 4. Four patients, one at the 400 mg/m^2 cloretazine dose level, one at 500 mg/m^2 cloretazine dose level, and two at the 600 mg/m^2 cloretazine dose level, achieved CR. Six patients, one at the 400 mg/m^2 cloretazine dose level, four at 500 mg/m^2 cloretazine dose level, and one at 600 mg/m^2 cloretazine dose level achieved CR$_P$. All patients achieving CR or CR$_P$ responded to the first course of therapy. Of seven patients with AML who achieved CR, four were previously treated with ara-C containing regimens, including three with primary refractory disease who had failed to achieve CR to induction therapy; the fourth patient has an initial CR duration of 31 weeks. Details of individual responses are shown in Table 5.

O^6-alkylguanine DNA alkyltransferase. Samples from 30 patients were analyzed for pretreatment AGT activity. Table 6 shows the AGT results sorted from lowest to highest activity. The median AGT level in peripheral blood mononuclear cells from leukemia patients was 8.61 fmol O^6-methylguanine per removal per μg DNA (range, 1.72-31.45). There was no correlation between alkyltransferase activity and age, gender, or the amount of circulating blasts. In addition, none of the samples had undetectable AGT levels.

The seven samples of responders (CR and CR$_P$) tended to have a lower median AGT activity (5.73 fmol O^6-methylguanine/μg) when compared with the activity in patients who did not respond to treatment (9.21 fmol O^6-methylguanine/μg, n = 23), although this was not statistically significant. Of the 15 samples in the group below the median AGT activity, five were from patients who responded to treatment and six were from patients treated at the 300 and 200 mg/m^2 dose level, where no drug activity was observed. Therefore, the AGT results were analyzed to include only patients treated at the ≥400 mg/m^2 dose levels as shown in Fig. 2. In this group, the mean AGT activity in responding patients is significantly lower than in nonresponding patients (6.7 and 13.9 fmol O^6-methylguanine/μg DNA, respectively; $P \leq 0.027$).

Discussion

The purpose of this phase I study was to define reasonable doses of cloretazine and ara-C that may be combined and examined in the phase II setting in patients with refractory leukemia. Cloretazine-based combinations are of interest because of the single-agent antileukemia activity and its chief toxicity of myelosuppression with no extramedullary DLTs at marrow ablative doses. This latter property suggests that combinations of cloretazine with other effective antileukemia agents may be feasible without additive or synergistic extramedullary toxicities. Ara-C is a logical choice to combine with cloretazine based on its established activity in patients with leukemia. Consistent with the established toxicities of ara-C, three DLTs observed on this study were gastrointestinal, none of which are seen with cloretazine as a single agent. The observation that cloretazine doses of 500 and 600 mg/m^2 given with a 4-day ara-C regimen were associated with these DLT, whereas the same cloretazine doses given with a 3-day ara-C regimen were not, further suggests that ara-C is responsible for most of the serious extramedullary toxicity seen with the combination. Grade 3 and 4 myelosuppression and fatigue observed on this study could reasonably be attributed to either drug within the combination.

The overall response rate of 27% seen in 10 (7 AML, 2 ALL, and 1 blastic phase chronic myeloid leukemia) of 37 evaluable patients is encouraging. Responses were only seen at dose levels of ≥400 mg/m^2. The overall study population had very advanced disease with 21 of 32 (66%) patients with AML receiving the study regimen as second or subsequent salvage. Of

Table 6. Pretreatment AGT activity and response to cloretazine and ara-C treatment

Patient no.	Diagnosis	Gender	Dose (mg/m^2)	Peripheral blasts (%)	AGT (fmol O^6-mG/μg DNA)	Response
7	AML	M	300	8	1.72	No response
3	AML	F	200	77	2.63	No response
16	AML	F	500	83	3.97	CR
5	ALL	F	200	88	4.10	PD
27	ALL	M	600	20	4.68	CR_P
6	MDS-AML	M	200	94	4.70	No response
22	MDS-AML	F	600	63	4.96	Early death
9	MDS-AML	M	300	26	5.05	No response
38	ALL	M	600	0	5.68	CR
25	MDS-AML	M	600	0	5.73	CR
10	AML	M	400	21	6.48	No response
28	AML	M	600	91	6.91	PD
11	AML	M	400	6	7.10	CR
8	ALL	M	300	90	7.96	PD
14	MDS-AML	M	500	57	8.28	No response
12	AML	F	400	96	8.94	CR_P
24	MDS-AML	F	600	49	9.05	Death
37	ALL	M	600	0	9.21	No response
15	AML	F	500	2	10.58	CR_P
21	MDS-AML	M	400	39	10.58	No response
17	ALL	M	500	12	11.38	No response
13	AML	F	400	24	11.52	No response
26	AML	M	600	12	11.86	PD
20	AML	F	400	3	12.93	No response
23	MDS-CMML	F	600	17	17.02	No response
1	AML	F	200	9	19.46	No response
39	MDS-AML	M	600	34	20.04	No response
40	AML	F	500	0	22.84	No response
41	MDS-AML	F	500	35	27.56	No response
36	MDS-AML	M	600	66	31.45	No response

Abbreviation: O^6-MG, O^6-methylguanine.

seven patients with AML who achieved CR, four were previously treated with ara-C containing regimens, including three with primary refractory disease who had failed to achieve CR to induction therapy; the fourth patient has an initial CR duration of 31 weeks. It seems likely that cloretazine made a significant contribution to attainment of CR in these patients. Three patients with previously untreated AML, who were considered unfit for standard induction regimens, achieved CR with the study regimen; it is difficult to specifically "assign" success to either drug in these patients, and these small numbers preclude comment on the tolerance of the study regimen in such patients relative to standard induction approaches. The relative roles of cloretazine and ara-C in the activity of the study regimen's activity should be evident when data is available from a recently initiated international multicenter study in patients with first relapse of AML who are randomized to receive the recommended phase II and III dose defined in this study versus the ara-C regimen alone. This opportunity to randomize patients to a high-dose ara-C regimen alone versus the same ara-C regimen with an investigational agent is rare in leukemia, as additive or synergistic toxicities have previously required a reduction in the ara-C dosage in the investigational arms of such randomized studies.

Two patients with refractory ALL achieved CR. This is noteworthy as both had resistant, intensively pretreated disease. Relapsed ALL remains a particularly poor prognosis disease and one in which ara-C is not particularly active. The role of cloretazine in lymphoid malignancies warrants further investigation. Phase I and II studies in patients with chronic lymphocytic leukemia and Richter's transformation are being conducted.

The study of resistance mechanisms may help in the development of cloretazine. A focus on AGT, which removes initial monoadducts in DNA and prevents cross-linking, seems warranted (16, 17, 30). Various types of tumors express higher levels of alkyltransferase when compared with normal tissue (30). The AGT results from this study are similar to previously published values for patients with first-relapse AML (31). On a prior study, cloretazine had no effect on AGT levels in patients treated with a 155 mg/m^2 thrice daily (total dose, 465 mg) regimen (22). In future studies, the measurement of AGT levels in "pure" blast population maybe of particular interest. In addition, this study showed that prestudy AGT levels vary in patients with leukemia, which might indicate differences in sensitivity to alkylators and subsequently potentially predict therapeutic outcomes. These data suggest that low AGT activity

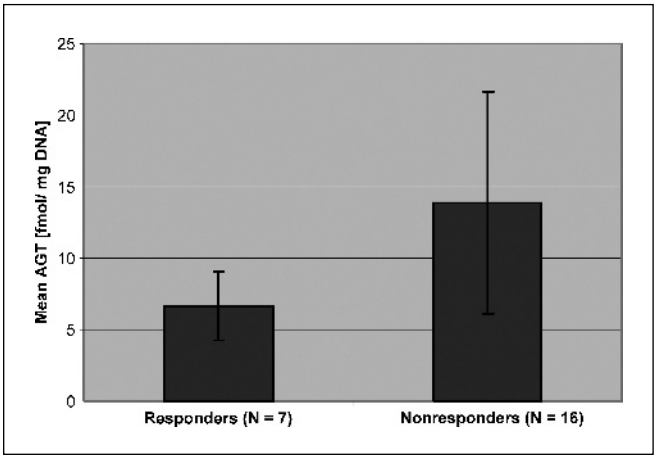

Fig. 2. AGT activity in peripheral blood mononuclear cells from patients who responded to cloretazine and those who did not in patients treated at dose levels $\geq$400 mg/m^2. Columns, mean; bars, $\pm$SE.

was associated with responsiveness to cloretazine, which raises the possibility that the depletion of AGT in tumor cells might augment the antitumor activity of cloretazine. If further studies confirm these finding, the use of cloretazine might be reserved for patients with leukemia and a low baseline alkyltransferase activity. Alternatively, it might be possible to increase the sensitivity of cloretazine with the use of agents that lower AGT levels.

O^6-benzylguanine has been shown to deplete tumor AGT in solid tumor clinical studies, but combinations of O^6-benzylguanine with BCNU or temozolomide have resulted in substantial hematologic toxicity at doses of the latter agents that are much lower than their single-agent MTD (25, 32, 33). Studies of O^6-benzylguanine in combination with cloretazine may be feasible in patients with leukemia, where myelosuppression may be desirable. Temozolomide, a methylating agent, has activity in relapsed leukemia, and standard regimens of temozolomide reduce AGT in tumor cells (34, 35). Resistance to temozolomide has been associated with both AGT overexpression and lack of DNA mismatch repair (36, 37). Combinations of temozolomide and cloretazine may warrant further investigation in relapsed leukemia, as temozolomide may deplete AGT and thus sensitize leukemia cells to cloretazine, neither agent has significant extramedullary toxicity, and mismatch repair deficiency does not seem involved in tumor resistance to cloretazine. A phase I study of temozolomide followed by cloretazine in patients with leukemia was recently initiated.

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