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ORIGINAL ARTICLE

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In vitro and in vivo effects of acetyldinaline on murine megakaryocytopoiesis

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Abstract *Purpose*: Acetyldinaline (CI-994) has shown preclinical efficacy in vitro and in vivo against solid tumor and leukemia cell lines. Since myelosuppression was the dose-limiting toxicity for acetyldinaline in preclinical and clinical studies, experiments were conducted to examine the in vitro and in vivo effects of acetyldinaline on murine megakaryocytic (CFU-meg) progenitor cells. *Methods*: Bone marrow and spleen cells from untreated mice were continuously exposed in vitro to acetyldinaline or dinaline in clonal assays. For the in vivo study, BDF_1 mice were dosed orally with 50 mg/kg acetyldinaline every day for 14 days. *Results*: Both acetyldinaline and dinaline induced an in vitro dose-dependent decrease in CFU-meg colonies derived from either the spleen or bone marrow. Splenic CFU-meg were more sensitive in vitro to acetyldinaline and dinaline than their marrow counterparts. In the in vivo experiments, platelet counts decreased throughout the 14-day dosing period and had returned to normal by day 18. Marrow and spleen CFU-meg declined after the first dose but had recovered by days 4 and 7, respectively. Elevated splenic CFU-meg counts were observed through day 20, 6 days after dosing ended. Recovery of platelet counts in treated mice was associated with increases in both marrow and splenic CFU-meg. *Conclusions*: There was differential in vitro toxicity of acetyldinaline to murine CFU-meg derived from the bone marrow versus spleen. The in vitro assay predicted the more severe effect of acetyldinaline on splenic progenitors than on their marrow counterparts that was observed in the in vivo phase. In addition, megakaryocytopoiesis in the marrow showed evidence of recovery from drug toxicity in the face of continuing daily acetyldinaline treatments.

Keywords Acetyldinaline · Mice · Platelets · CFU-meg · In vitro · In vivo

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Introduction

Acetyldinaline (4-acetylamino-*N*-[2′-aminophenyl]-benzamide, CI-994, PD-123654), an acetylated derivative of dinaline (GOE-1734, PD-104208), has shown preclinical efficacy in vitro and in vivo against solid tumor and leukemia cell lines [14]. In a rat model of acute myeloid leukemia, acetyldinaline demonstrated greater than 8-log leukemic cell kill with only 1-log cell kill of hematopoietic stem cells (CFU-s) [1]. Acetyldinaline has demonstrated activity against pancreatic, colon, mammary, prostate and osteogenic solid tumors and substantial log cell kill at the highest nontoxic doses. Prolonged administration of acetyldinaline at lower doses is more effective than short-term administration at higher doses [14]. In a rat model of chemically induced colorectal carcinoma, acetyldinaline showed high anticancer activity and lower mortality than dinaline and methyldinaline [19].

Acetyldinaline is of interest since its mechanism of action is different from more traditional cytotoxic anticancer mechanisms [13]. Kraker et al. have found that acetyldinaline arrests HCT-8 human colon adenocarcinoma cells in G_1 phase in a time- and concentration-dependent manner [10]. There was also a reduction in cellular pyrimidines that corresponded to both growth

delay and cell-cycle effects [10]. The authors observed that acetyldinaline inhibits histone deacetylase in enzyme assays and induces histone hyperacetylation in HCT-8 cells [11].

The preclinical pharmacology and toxicity of acetyldinaline, and its deacetylated metabolite, have been studied in female BDF_1 mice treated orally (50 mg/kg) once daily for 14 days [2]. Maximum plasma concentration (C_{max}) reached 20.63 and 22.12 μg/ml on the first and last days of dosing, respectively, with a decrease in peripheral blood counts for neutrophils, lymphocytes and platelets. The plasma concentrations of dinaline on days 1 and 14 of dosing were 0.738 and 1.00 μg/ml, respectively [2]. The platelet nadir (71% of control) occurred on day 7 with recovery by day 9, whereas the neutrophil nadir (38% of control) followed on day 9 and recovery on days 16–27 [2]. In another study of once-daily dosing for 2 weeks in rats (1.5–15 mg/kg) and dogs (0.5–5 mg/kg), myelotoxicity was manifested by lymphopenia, neutropenia, thrombocytopenia and bone marrow hypoplasia at the medium and high doses [5]. In the rat, there was splenic hematopoietic depletion at all doses, and myeloid and megakaryocyte hyperplasia at 2 mg/kg in the dog [5]. Thus, the preclinical studies have shown that acetyldinaline's dose-limiting toxicity is reversible bone marrow suppression.

A phase I clinical trial using chronic oral administration of acetyldinaline (5–15 mg/m^2) confirmed the animal pharmacology with C_{max} values ranging from 168 to 570 ng/ml [18]. The maximum tolerated dose was 8 mg/m^2 per day for 8 weeks with thrombocytopenia as the dose-limiting toxicity. Grade 3 thrombocytopenia was noted in both the acute (2 weeks) and chronic (8 weeks) schedule at 15 mg/m^2 per day and 8 mg/m^2 per day, respectively [18]. The median time to nadir was 16 days and recovery occurred after withdrawal within 8–18 days. Myelotoxicity did not worsen with continued daily dosing. There was a direct relationship between plasma area under the curve (AUC) and thrombocytopenia which was not seen with neutropenia. Neutropenia did not exceed grade 2 and there was some grade 3 anemia at 5 mg/m^2 per day. Some instances of stable disease were also noted in this trial suggestive of acetyldinaline's clinical activity [18].

The objectives of this study were to evaluate the direct in vitro toxicity of acetyldinaline and dinaline on megakaryocytic (CFU-meg) progenitors from murine marrow and spleen at pharmacologically relevant concentrations and the relationship between CFU-meg and peripheral platelet counts in the marrow and spleen of treated mice during chronic daily dosing. The two phases allowed for in vitro–in vivo correlations in mice and comparison with toxicity noted in clinical trials.

Materials and methods

Stock solutions of acetyldinaline and dinaline were prepared in ethanol and were then diluted in Iscove's modified Dulbecco's medium (IMDM) plus 20% fetal bovine serum (FBS) to ten times the desired final concentration. Acetyldinaline and dinaline were added to final concentrations of 0.03–24 and 0.009–3.0 μg/ml, respectively, directly to the marrow or spleen cells in the in vitro clonal assays (continuous exposure). Vehicle control groups were treated with an equal amount of ethanol (up to 1%) in IMDM. A stock solution of acetyldinaline (in ethanol) was diluted to 0.5 and 1.5 μg/ml in IMDM plus 20% FBS and incubated at 37°C for 2 weeks. The stability of acetyldinaline in culture medium was assessed by reverse-phase (RP) HPLC following solid-phase extraction [2] and found to be stable for up to 14 days under culture conditions.

All experiments were performed with female BDF_1 ($C_{57}Bl/6\times DBA_2$) mice under protocols approved by the Wayne State University (in vivo) and FDA Center for Drug Evaluation and Research (in vitro) animal care and use committees employing NIH guidelines. For the in vivo experiments, the mice were orally dosed with an acetyldinaline suspension in ethanol (up to 5%) and distilled water at 50 mg/kg (150 mg/m^2) daily for 14 days. Vehicle control mice were given identical volumes of distilled water. On designated days, drug-treated and vehicle control mice (three to five per group) were killed, and femurs, spleens and peripheral blood collected. Bone marrow cells were flushed from femurs with a small-gauge needle and aspirated several times to achieve a single-cell suspension. Spleens were teased apart using sterile needles into small cell clumps, followed by repeated aspiration through a pipette to obtain a single-cell suspension. Mononuclear spleen cells were separated by gradient centrifugation with Ficoll-Paque (Pharmacia, Piscataway, N.J.). Platelets were quantified from blood smears. Marrow and spleen cells were harvested from treated mice and cultured in the CFU-meg assay as described below in the absence of drug or vehicle.

Megakaryocyte colonies were grown in fibrin clots for 6 days in 35-mm culture dishes [12]. The clots contained 20% FBS, 10% pokeweed mitogen spleen-conditioned medium [7], 20% double-strength IMDM, $1.5–7.0\times10^5$ marrow or spleen cells/ml, vehicle or drug to desired concentration, 0.5 mg/ml bovine fibrinogen and 0.5 U/ml bovine thrombin. Each dish contained 0.4 ml of this mixture to which 0.6 ml IMDM was added after clotting. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. CFU-meg colonies were stained for acetylcholinesterase activity [16] and aggregates of four or more positive cells were scored as colonies.

The percent colony inhibition in the in vitro experiments was determined by comparing plating efficiency (colonies per 10^5 nucleated cells) of drug-treated groups to that of the vehicle (untreated) controls. IC_{50}, IC_{70} and IC_{90} values were calculated by a linear regression analysis of the concentration-inhibition curves. Platelet counts and colony numbers from drug-treated mice were compared to those from paired vehicle controls from the same day of the study.

Results

In vitro experiments

The plating efficiencies (colonies/10^5 cells) for controls were 17 ± 6 and 2 ± 1 for CFU-meg derived from femur marrow and spleen cells, respectively ($n=8$ plates). The acetyldinaline concentrations in the in vitro experiments were chosen to correlate with in vivo C_{max} (20.63–22.12 μg/ml) and trough (0.05–0.2 μg/ml) levels in mice [2]. Both acetyldinaline and dinaline induced a concentration-dependent decrease in CFU-meg colonies derived from either the spleen or bone marrow (Fig. 1), from which the inhibitory concentrations were derived (Table 1). Splenic CFU-meg were more sensitive in vitro

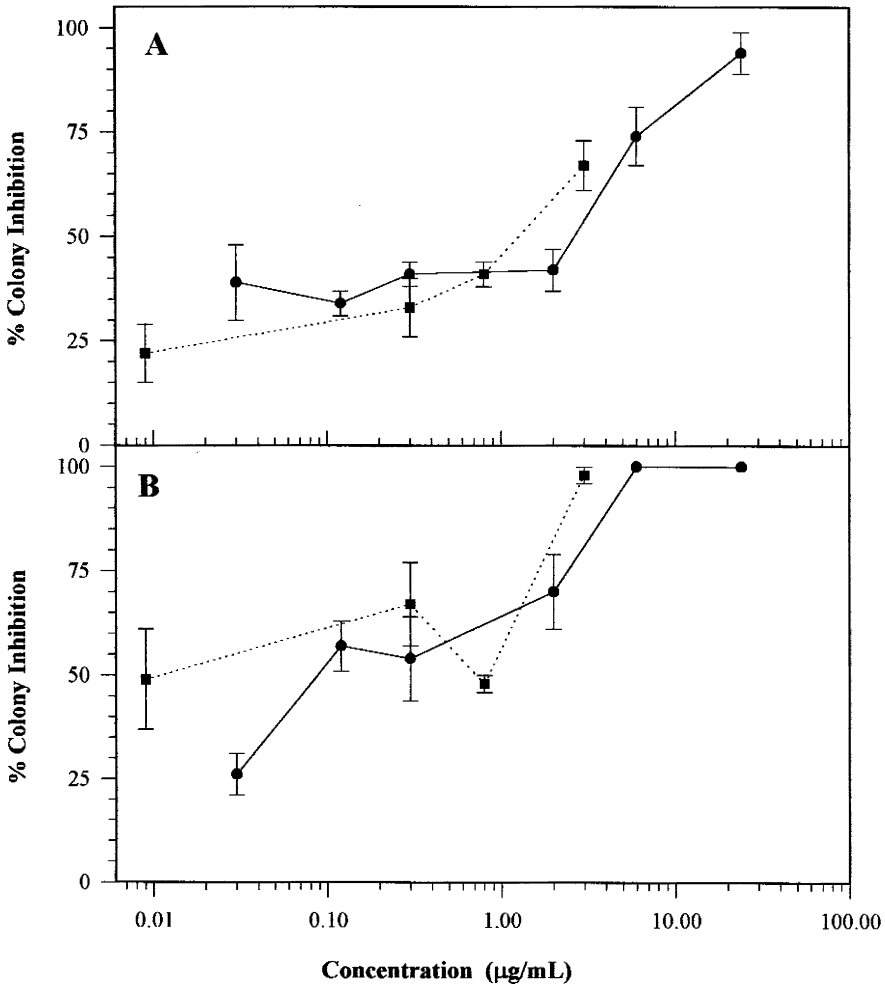

Fig. 1A, B In vitro effects of acetyldinaline and dinaline on murine marrow (**A**) and spleen (**B**) CFU-meg (● acetyldinaline, ■ dinaline). The values are means ± SE of four dishes

Table 1 In vitro inhibitory concentrations for CFU-meg colonies. IC_{50}, IC_{70} and IC_{90} values (μg/ml) were calculated by linear regression

Source	Drug	IC_{50}	IC_{70}	IC_{90}
Marrow	Acetyldinaline	0.51	5.60	61.63
	Dinaline	1.10	>3.0[a]	>3.0[a]
Spleen	Acetyldinaline	0.16	1.00	6.25
	Dinaline	0.02	0.58	14.49

[a]Inhibition <70% or <90% at 3.0 μg/ml dinaline

to acetyldinaline and dinaline than their marrow counterparts. In the bone marrow, acetyldinaline was less toxic than dinaline, whereas in the spleen, the two compounds were more similar in their colony inhibition.

In vivo experiments

In the paired vehicle control mice, the number of CFU-meg ranged from 1407 to 14,463 in the femur and from 109 to 1086 in the spleen. After the 1st and 4th day of oral acetyldinaline dosing (50 mg/kg), marrow CFU-meg levels were 64% and 132%, respectively, of the paired vehicle control levels (Fig. 2A). Marrow CFU-meg had decreased by day 9 although the levels were above control levels again on day 16. On day 1, splenic CFU-meg levels were 3% of the paired vehicle control levels, and steadily increased to 639% of control levels (Fig. 2A). The splenic CFU-meg content decreased through day 20 to 234% of control levels. In summary, splenic CFU-meg were more reduced than femoral progenitor levels initially after dosing began, but were elevated to a greater degree by the 2nd week of dosing and thereafter.

Platelet counts in the paired control mice ranged from 752 to 1129×10^6/ml. The platelet counts decreased in the treated mice through day 7 to 37% of the paired vehicle control counts, which was followed by an increase through day 11 to 84% of the control counts (Fig. 2B). After the drug was discontinued, the platelet counts had returned to normal by day 18 and were 119% of control counts on day 27. The platelet count was declining at the same time marrow and splenic CFU-meg were beginning to recover from their initial toxicity. After this increased progenitor level, the platelet count began to return to control levels. While the CFU-meg levels in the bone marrow fluctuated, splenic CFU-meg levels remained greatly elevated to sustain this platelet recovery.

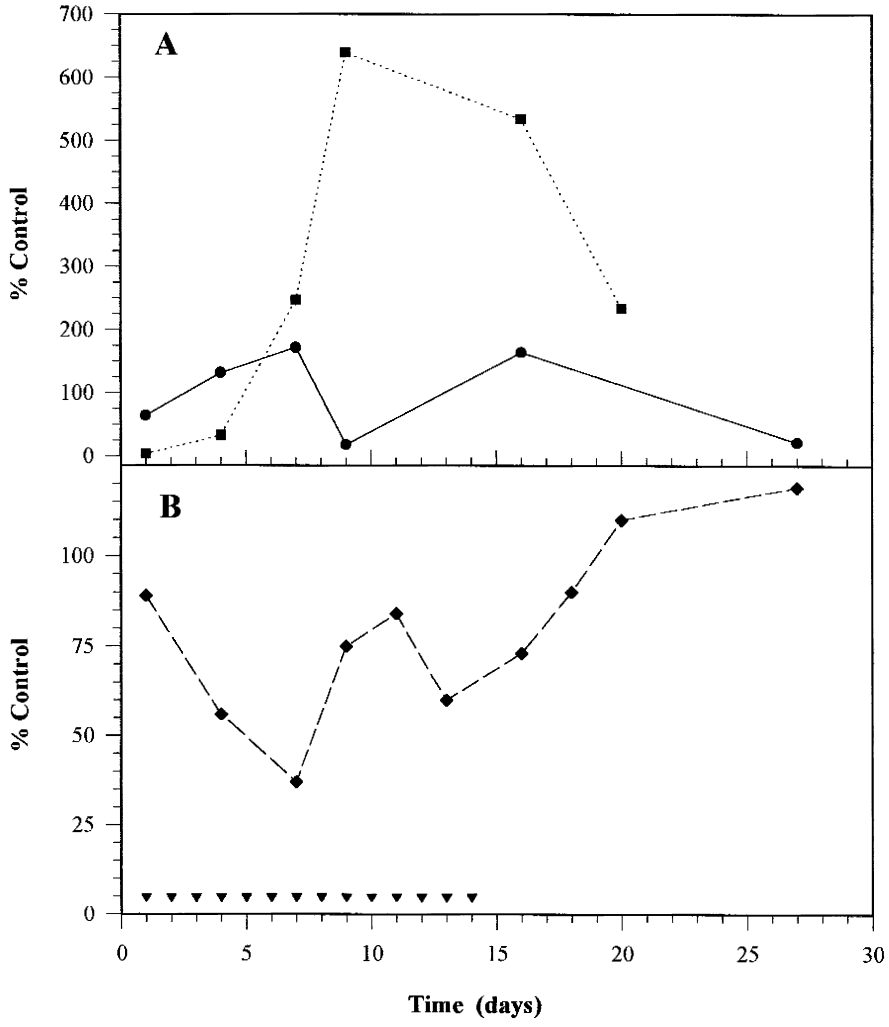

Fig. 2A, B In vivo effects of acetyldinaline on murine CFU-meg content (**A**) and platelet counts (**B**) compared to paired vehicle controls (● marrow CFU-meg, ■ splenic CFU-meg, □ platelets) following oral dosing (50 mg/kg) on days 1 through 14 (▼). The results shown are the means from three to five mice

Discussion

Thrombocytopenia is the dose-limiting toxicity in clinical studies for both acute and chronic dosing [15, 18]. This was not unexpected based on the previous preclinical studies in mice [2] and rats [6]. The results presented demonstrate that acetyldinaline and its parent, dinaline, induced a concentration-dependent decrease in megakaryocyte progenitors from murine bone marrow and spleen following in vitro drug exposure. Splenic CFU-meg were more sensitive than marrow CFU-meg to acetyldinaline and dinaline when exposed during the 6-day in vitro culture period (Fig. 1). When compared to the plasma concentration (20 μg/ml) achieved in mice (50 mg/kg per day), approximately 90% and 100% of marrow and spleen CFU-meg colony formation was inhibited following a 6-day drug exposure. In the in vitro studies, CFU-meg from the spleen were more sensitive to acetyldinaline than their marrow counterparts (Table 1). These in vitro results correlated with those of the in vivo study in that the splenic progenitor nadir was initially more severe than that of CFU-meg in the marrow. However, the rebound increase in splenic CFU-meg exceeded that of the bone marrow in mice dosed with acetyldinaline.

Furthermore, it appears that very high numbers of splenic CFU-meg can increase platelet counts, but cannot completely compensate for loss of megakaryocytes in the marrow after a 14-day acetyldinaline dosing period. However, recovery of CFU-meg levels with continued daily therapy is due to increased repopulation activity in the stem cell compartments that compensates for drug toxicity to progenitors [8, 9]. Treatment with acetyldinaline for 10 consecutive days resulted in a modest effect on murine short-term repopulating cells but spared long-term repopulating cells. In the mice, acetyldinaline did not cause severe permanent damage to the hematopoietic system [9, 10]. Continued daily dosing in a phase I trial with acetyldinaline did not worsen the myelotoxicity, which was reversible within 1 days after discontinuing therapy [15].

Granulocyte progenitors (CFU-gm) have also been evaluated following both in vitro and in vivo drug exposure [20]. Marrow CFU-gm were more resistant to the toxic effects of acetyldinaline and dinaline than

those for the spleen as we had seen with CFU-meg. The IC_{70} value was higher for femoral CFU-meg (5.60 μg/ml) than CFU-gm (1.7 μg/ml) with no great differences for the two progenitors from the spleen. White blood counts and neutrophils in mice decreased through 9 days of a 14-day dosing period and had reached control levels by day 20. The femoral CFU-gm content fell below control levels on day 1 and was normal by day 4. Splenic CFU-gm showed decreases through day 4 and had rebounded by day 7 [20]. As with the platelets, improvement of leukocyte and neutrophil counts were related to increases of CFU-gm in the spleen [20].

Other investigators have noted a greater response of splenic progenitors (granulocytic, erythroid, megakaryocytic) as compared to the bone marrow in mice treated with cytotoxic drugs [3, 4]. Mice given a single dose of 5-fluorouracil (150 mg/kg) exhibited moderate thrombocytopenia on day 7 with a rebound on days 11 to 17 [20]. There was a decrease in marrow CFU-meg on day 2 and on day 5 in the spleen. There was a rebound increase in CFU-meg in the spleen on days 11 to 17, but not in bone marrow . The simultaneous maximal thrombocytosis and increased splenic CFU-meg suggests that increased platelet production after dosing with 5-fluorouracil is associated with stimulation of the splenic megakaryocyte progenitor compartment [21]. Cyclophosphamide produced a mild thrombocytopenia in mice (platelets 88–95% of control levels) with thrombocytosis on day 10 (120%) [17]. Megakaryocytes and CFU-meg were 10–25% of the control levels within 24 h of administration. This was followed by recovery of spleen megakaryocytes and CFU-meg to normal levels within 6 days, then a 24- and 29-fold increase in splenic megakaryocytes and CFU-meg, respectively. In contrast, recovery of megakaryocytes and CFU-meg in the marrow of treated mice was delayed for 2 weeks [17].

Our in vitro and in vivo murine studies show that spleen-derived megakaryocytic progenitors plays an important role in the hematological recovery from acetyldinaline's thrombocytopenic effect in treated mice, which is augmented by a slower recovery of the marrow CFU-meg pool. The data also show that dinaline could conceivably contribute to thrombocytopenia in the mouse if its plasma concentration after acetyldinaline dosing reaches IC_{70} to IC_{90} levels. When comparing these murine results with those of a phase I clinical trial [18], toxicity of acetyldinaline to CFU-meg (in vitro) or platelets (in vivo) is concentration- and AUC-dependent, respectively. Also of note was that the thrombocytopenia that resulted from chronic acetyldinaline dosing did not worsen in patients, and actually improved through dosing in mice. This study shows that investigators can learn more about the myelotoxicity of new chemotherapeutic agents and their metabolites with clonal assays following in vitro or in vivo drug exposure.

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