Pharmacological and Biochemical Strategies to Increase the Accumulation of Arabinofuranosylguanine Triphosphate in Primary Human Leukemia Cells

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ABSTRACT

Purine nucleoside phosphorylase deficiency leads to a dGTP-mediated T-lymphopenia, suggesting that an analogue of deoxyguanosine would be selectively effective in T-cell disease. 9-β-D-Arabinofuranosylguanine (ara-G) is relatively resistant to hydrolysis by purine nucleoside phosphorylase and selectively toxic to T cells, but its low solubility has prevented its use in the clinic. 2-Amino-6-methoxyarabinofuranosylpurine (GW506U) serves as the water-soluble prodrug for ara-G. A Phase I trial in patients with refractory hematological malignancies demonstrated that the clinical responses to this agent were directly related to the peak bevels of ara-G 5'-triphosphate (ara-GTP) in target cells. The aim of the present study was to develop and test strategies to increase intracellular accumulation of ara-GTP in primary human leukemia cells of myeloid and B-lymphoid origin. Three strategies were tested. First, incubations with 100 μM ara-G for 4 h produced a linear median accumulation rate of 19 μM/h (range, 2–45 μM/h; n = 15) in lymphoid leukemia cells and 16 μM/h (range, 0.5–41 μM/h; n = 11) in myeloid leukemia cells. Saturation of ara-GTP accumulation was achieved only after 6–8 h exposure in both lymphoid and myeloid leukemia cells, suggesting a rationale for prolonged infusion. Second, a dose-dependent increase in ara-GTP accumulation was observed with incubations of 10–300 μM ara-G for 3 h. Hence, dosing regimens that achieve high plasma levels of ara-G during therapy may increase cellular levels of ara-GTP. Finally, a biochemical modulation approach using in vitro incubation of leukemia cells with 10 μM 9-β-D-arabinofuranosyl-2-fluoroadenine for 3 h, followed by either 50 or 100 μM ara-G for 4 h, resulted in a statistically significant median 1.3-fold (range, 1.1–9.0-fold; P = 0.034) and 1.8-fold (range, 0.9–10.6 fold; P = 0.018) increase in ara-GTP compared to cells incubated with ara-G alone. Extension of these studies to ex vivo incubations confirmed our in vitro findings. These strategies will be used in the design of clinical protocols to increase ara-GTP accumulation in leukemia cells during therapy.

INTRODUCTION

Deficiencies in adenosine deaminase (EC3.5.4.4) and PNP (EC2.4.2.1) result in two naturally occurring immune diseases (1, 2). In contrast to the severe combined immune deficiency seen with adenosine deaminase deficiency, PNP deficiency produces abnormalities only in T-cell-mediated immunity (2). In vitro studies with T cells from mice genetically deficient in PNP have suggested that deoxyguanosine is the only PNP substrate that causes the exquisite T-cell-directed toxicity (3), resulting in T-lymphopenia. Thus, development of either an inhibitor of this enzyme or of a PNP-resistant nucleoside, the triphosphate of which mimicked the cytotoxicity of dGTP, would be rational approaches to the treatment of leukemias in general and T-cell leukemias in particular.

ara-G, first synthesized in 1964 (4), has been shown to be relatively resistant to phosphorolysis by PNP (5). However, its limited solubility in water prevented its use in the clinic until recently, when a water-soluble prodrug, GW506U, was synthesized. This nontoxic precursor is demethoxylated by adenosine deaminase to ara-G, which has been shown to be effective in selectively killing T-cells (5–7). As is the case for other nucleoside analogues, the effectiveness of ara-G is thought to be due to cellular accumulation of ara-GTP. In vitro work using human leukemia cell lines (6, 8, 9) and primary leukemia cells obtained from patients (7) demonstrated that the differential accumulation of ara-GTP, the active metabolite, might be the mechanism for the selective sensitivity of T cells to this drug.

The initial and rate-limiting step in accumulation of ara-GTP from ara-G is the formation of 9-β-D-arabinofuranosylguanine 5'-monophosphate. This conversion is catalyzed by both

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3 The abbreviations used are: PNP, purine nucleoside phosphorylase; ara-G, 9-β-D-arabinofuranosylguanine; dGTP, deoxyguanosine triphosphate; dCK, deoxycytidine kinase; ALL, acute lymphoid leukemia; AML, acute myelogenous leukemia; CLL, chronic lymphoid leukemia; CML, chronic myelogenous leukemia; blast crisis; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-ATP, F-ara-A 5'-triphosphate; ara-GTP, ara-G 5'-triphosphate; fludarabine, F-ara-A 5'-monophosphate; GW506U, 2-amino-6-methoxypurine arabinoside.
dGK (10–12) and dCK (13–15). Compared to other cell types, T cells possess high activities of dGK and dCK (13) and little cytosolic nucleotidase activity (15), which would otherwise dephosphorylate ara-GTP. In a recently completed Phase I trial, GW506U cytotoxicity in hematological malignancies was correlated with the cellular accumulation of ara-GTP in circulating leukemia cells. The trial had a remarkable clinical outcome: most patients with hematological malignancies involving immature T-lymphoid cells (T-ALL, CML-BC T-lymphoid, and T-lymphoma) achieved complete or partial remission (16).

Cellular pharmacokinetic studies during the Phase I trial demonstrated that ara-GTP was the major cellular metabolite after GW506U infusion (17). Correlation of the cellular pharmacokinetics and clinical response revealed that patients who achieved a complete or partial remission accumulated significantly higher peak ara-GTP levels (17) compared to nonresponders. The elimination of ara-GTP, on the other hand, was slow in patients regardless of diagnosis, which included T-ALL, AML, B-CLL, B-ALL, T-CLL, and normal mononuclear cells.

Taken together, these data clearly demonstrate the importance of the intracellular accumulation of ara-GTP in achieving clinical responses. These observations support the hypothesis that increasing the intracellular level of ara-GTP in target cells would result in clinical responses in non-T as well as T-cell hematological malignancies. The aim of the present study was to design and evaluate pharmacological and biochemical strategies to increase intracellular ara-GTP during in vitro incubations of primary human leukemia cells. We propose ways to modify GW506U infusion based on our results.

PATIENTS AND METHODS

Patients. Twenty-six patients diagnosed with seven different hematological malignancies were studied; their characteristics are given in Table I. All blood samples were collected from patients at the University of Texas M. D. Anderson Cancer Center between May 1996 and February 1997, after obtaining informed consent. Patients were selected based on the high number of circulating leukemia cells in the peripheral blood (>8000/μl) and their willingness to provide samples for these studies.

Chemicals. Ara-G was initially purchased from Calbiochem (La Jolla, CA) and then from R. I. Chemical, Inc. (La Jolla, CA). F-ara-A was produced by alkaline phosphatase treatment of fludarabine, which was obtained from Berlex Laboratories (Richmond, CA). F-ara-ATP and ara-GTP were chemically synthesized by Sierra Bioresearch (Tucson, AZ). All other chemicals were reagent grade.

Peripheral Blood Leukemic Cells. Before therapy, 40-ml blood samples were collected in heparinized tubes from each patient. The tubes were immediately placed in an ice water bath and then transported to the laboratory for processing. Control studies have demonstrated that leukemia cells are stable under these conditions with respect to size, membrane integrity, and cellular nucleotide content for at least 15 h (18). The blood samples were diluted with PBS (8.1 g of NaCl, 0.22 g of KCl, 1.14 g of Na2HPO4, and 0.27 g of KH2PO4/liter H2O, pH 7.4.), and mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation procedures as described previously (19).

The cells were then washed with PBS and resuspended in warmed RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and maintained at 37°C in 5% CO2 in a fully humidified incubator. Cell number and size were determined with a Coulter counter (Coulter Electronics, Hialeah, FL).

In Vitro Incubations with ara-G. Leukemia cells were incubated with the indicated concentrations of ara-G. Incubations were maintained for 3–8 h, and aliquots (5 × 106–2 × 108 cells) were removed at hourly intervals. The nucleotides were extracted and quantitated as described below.

In Vitro Incubations with F-ara-A and ara-G. For the in vitro combination studies, cells obtained before therapy were incubated for 3 h either with no drug or with 10 μM F-ara-A. After that time, the cells were washed in three volumes of warmed drug-free culture medium and then resuspended in fresh medium to which the indicated concentration of ara-G was added. Samples were collected and processed at 0 h (to quantitate F-ara-ATP concentration) and at 1, 2, 3, and 4 h to determine intracellular ara-GTP levels as described below.

Ex Vivo Studies with Fludarabine and ara-G. For these investigations, leukemia cells were obtained from patients with AML or B-CLL before (15 or 30 mg/m2 infusion of fludarabine, respectively) and 4 h after therapy. The cells were isolated as described above and incubated separately, with the indicated concentrations of ara-G for 4 h. Samples were collected and processed as described above at 0 h (to quantitate F-ara-ATP concentration) and at 1, 2, 3, and 4 h to determine intracellular ara-GTP levels as described below.

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* Patients were grouped by diagnosis.
* PLL, ploymyphocytic leukemia.
* NR, not reported.
Measurement of Intracellular Nucleoside Triphosphates by High-Pressure Liquid Chromatography. Nucleotides were extracted by perchloric acid, and extracts were neutralized with KOH as described (20) and stored at −20°C until analyzed. The neutralized extracts were applied to a 10-SAX Partisil anion-exchange column (Waters Corporation) and eluted at a flow rate of 1.5 ml/min with a 50 min concave gradient (curve #7, Waters 600E System Controller; Waters Corporation, Milford, MA) from 60% 0.005 M gradient (curve #7, Waters 600E System Controller; Waters eluted at a flow rate of 1.5 ml/min with a 0.75 mm concave until analyzed. The neutralized extracts were applied to a 10-

Results

Influence of Increased Time of Incubation with ara-G on ara-GTP Accumulation. To determine if the rate of ara-GTP accumulation was linear with time, leukemia cells from patients were incubated with 100 µM ara-G for 4 h (Fig. 1). This concentration of ara-G was selected because a recent Phase I trial indicated that at the maximally tolerated dose of the ara-G prodrgu, GW506U, adults with leukemia achieved a median 116 µM plasma ara-G concentration (17). The data in leukemia cells of lymphoid origin from a total of 15 patients (Fig. 1, A and B) showed heterogeneous rates of accumulation of intracellular ara-GTP (median rate, 19 µM/h; range, 2–45 µM/h). At 4 h, the median intracellular ara-GTP concentration was 74 µM (range between 9 and 181 µM). Although there was heterogeneity among patients with respect to the absolute concentration of ara-GTP, in most cases (cells from 13 of 15 patients), the rate of accumulation of ara-GTP was linear, suggesting that a longer duration of incubation would result in a greater and continued accumulation of ara-GTP. In cells from 2 of the 15 patients studied (patients 12, 14), saturation of ara-GTP accumulation occurred within the first hour. To determine the extent of linear accumulation of ara-GTP, the time of exposure was increased to 8 h (Fig. 1B). Although there was a time-dependent increase in ara-GTP levels, the accumulation reached a plateau in the cells by 6–8 h in five of six patients tested. Ara-GTP accumulation did not appear to reach saturation in cells from one patient by 8 h (patient 1).

The data from leukemia cells of myeloid origin from a total of 8 patients (Fig. 1, C and D) also showed heterogeneity with regard to the rate of accumulation of intracellular ara-GTP (median rate, 16 µM/h; range, 0.5–41 µM/h). At 4 h, the median intracellular ara-GTP concentration was 41 µM (range, 2–166 µM). As was true for the lymphoid cells, the rate of accumulation of ara-GTP was generally linear for 4 h, suggesting that a longer duration of incubation would result in greater and continued accumulation of ara-GTP. Longer exposure time (8 h of incubation) suggested a time-dependent increase in ara-GTP levels; the accumulation reached a plateau in the cells by 6–8 h in 2 of 4 patients tested (Fig. 1D). Compared with lymphoid cells (median level at 4 h, 74 µM), myeloid cells (median level at 4 h, 41 µM) accumulated lower ara-GTP levels. Although there was variation both within and between diagnoses with respect to saturation of ara-GTP accumulation in vitro, these data suggest that increasing the duration of exposure to ara-G also may increase the intracellular levels of ara-GTP accumulated by leukemia cells during therapy.

Influence of Increased Concentration of ara-G on ara-GTP Accumulation. To determine the relationship between the concentration dose of ara-G and ara-GTP accumulation, leukemia cells were exposed to increasing ara-G levels for 3 h (Fig. 2). In 8 of 17 patients (patients 1–3, 14, 17–19, and 25), incubation with up to 300 µM ara-G resulted in a proportional increase in ara-GTP accumulation. In 5 of 17 patients (patients 9, 11, 13, 15, and 26), the accumulation of ara-GTP appeared to saturate at 200 µM. In the remaining 3 of 17 patients, saturation...
Strategies to Increase ara-GTP Accumulation in Leukemia Cells

Fig. 2 Dose-dependent accumulation of ara-GTP in primary leukemia cells. Peripheral blood mononuclear cells were obtained from patients with: A, B-CLL (n = 4); B, B-ALL (●, n = 2); B-PLL (■, n = 1); or T-PLL (▲, n = 1); C, AML (n = 7); and D, CML-BC (n = 2). The leukemia cells were washed and incubated with various concentrations of ara-G for 3 h, extracted with perchloric acid, and analyzed as described in “Patients and Methods.” Each symbol represents a different patient.

of accumulation occurred by 100 µM (patients 16, 20, and 23) or was too low to evaluate adequately (patient 24). These data demonstrate that increasing the concentration of exogenous ara-G above 100 µM generally resulted in an increased intracellular accumulation of ara-GTP. The median peak plasma concentration of ara-G in 42 patients studied in a Phase I trial of GW506U occurred at the end of the 1-h infusion (17). Hence, a more rapid infusion rate of a given GW506U dose would result in a higher concentration of peak plasma ara-G and intracellular ara-GTP. Alternatively, increasing the daily dose of infusion, with appropriate compensation for toxicity by administration of fewer than the 5 doses given in the original trial (16), may accomplish this goal.

Influence of F-ara-A Preincubation on ara-GTP Accumulation in Vitro and ex Vivo. The rate-limiting step in the conversion of ara-G to ara-G monophosphate is likely to be catalyzed most efficiently by dGK at low concentrations of substrate (K_m = 7–65 µM; Refs. 5, 12, 21, and 22) and by dCK at higher concentrations of ara-G (K_m = >100 µM; Refs. 5 and 23). The activities of dCK and dGK are feedback inhibited by dCTP and dGTP, respectively (10, 24–28). Fludarabine was shown to decrease the dCTP and dGTP pools to 40 and 70% of pretreatment levels (28). This decrease in regulatory deoxynucleotide triphosphates may effectively alleviate their feedback inhibition of the kinases that activate ara-G. To evaluate the modulation of ara-GTP accumulation in vitro by the nucleoside of fludarabine, F-ara-A, leukemia cells were incubated with 10 µM F-ara-A for 3 h and then incubated with 50 µM ara-G or 100 µM ara-G for up to 4 h (Fig. 3). As predicted from the concentration dependency studies presented above, the rate of accumulation was different at the different ara-G concentrations. At each concentration, however, there was an approximately 2-fold increase in ara-GTP accumulation for up to 4 h of incubation. The F-ara-ATP level at the time of ara-G addition in this patient was 319 µM. The results from the cells of 12 patients that were incubated in this fashion are summarized in Table 2. Preincubation of the primary leukemia cells with F-ara-A resulted in a median F-ara-ATP concentration of 127 µM (range, 18–383 µM; n = 12). The modulation of the ara-GTP accumulation by F-ara-A (expressed as a fold change relative to fludarabine naïve cells) at 50 and 100 µM ara-G for 3 h resulted in a median fold change of 1.3 and 1.8, respectively (range: 1.1–9.0, n = 8, and 0.9–10.6, n = 12, respectively), which was statistically significant at both concentrations (50 µM, P = 0.034; 100 µM, P = 0.018).

To extend these in vitro observations, we determined if a similar modulation of ara-GTP accumulation could be achieved ex vivo. Data obtained from one patient with AML are shown in Fig. 4 for illustrative purposes. These data suggest that the intracellular F-ara-ATP concentration achieved after fludarabine infusion (20 µM) was sufficient to modulate ara-GTP accumulation at all concentrations of ara-G tested (Fig. 4A) and for incubations of up to 4 h with 100 µM ara-G (Fig. 4B). For a total of three patients (two AML and one B-CLL), there was a 1.2-, 1.8-, and 4.2-fold increase in ara-GTP accumulation 4 h after the end of fludarabine infusion. This was a significant increase (P = 0.030) at all concentrations of ara-G tested. These data suggest that an infusion of fludarabine followed 4 h later by GW506U might result in an increased accumulation of ara-GTP in target cells.
in the target cells during therapy. To establish rationales for the design of prospective clinical protocols, we evaluated two pharmacological strategies and one biochemical approach to increase the intracellular accumulation of ara-GTP during in vitro incubations of primary non-T leukemia cells.

The first pharmacological strategy tested the effect of prolonging the duration of exposure to ara-G on intracellular ara-GTP. Because the peak plasma concentration of ara-G was 116 μM at the maximally tolerated dose (17), we determined if ara-GTP accumulation was linear with time at such concentrations. The data presented in Fig. 1 in cells of both lymphoid and myeloid origin demonstrated heterogeneity among patients with respect to the absolute concentration of ara-GTP, but in the majority of cases, the rate of accumulation was linear for the first 4 h. We then determined for how long the linearity of ara-GTP accumulation persisted. Although there was a time-dependent increase in the ara-GTP level, the accumulation reached a plateau by 6–8 h in both lymphoid and myeloid cells. These data suggest that prolonging the duration of exposure to ara-G by increasing the duration of infusion may result in a greater and continued intracellular accumulation of ara-GTP by leukemia cells. This strategy is being tested clinically in a Phase I trial with GW506U in escalating doses but as a single 4-h infusion. We expect that the reduction of the dose frequency to one per course will permit administration of a greater amount of the drug per infusion. This study design should expose leukemia cells to a 4-fold greater duration of exposure of maximal plasma ara-G concentration compared to the initial Phase I trial.

The purpose of the second pharmacological strategy was to determine the ara-G dose dependency of ara-GTP accumulation. Increasing the concentration of exogenous ara-G to 300 μM for 3 h resulted in a proportional increase in the accumulation of intracellular ara-GTP in the leukemia cells from the majority of the patients (Fig. 2). In contrast, the maximum rate of triphosphate accumulation is achieved at relatively low concentrations (5–20 μM) of ara-C (32–34) and gemcitabine (33–34), reflecting the affinity of dCK for these pyrimidine nucleoside analogues (34). On the other hand, purine analogues, such as fludarabine

### Table 2 In vitro modulation of ara-GTP accumulation by preincubation with F-ara-A

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*Pre F and Post F, without or with a 3-h incubation with 10 µM F-ara-A, respectively.

Fig. 4 Ex vivo modulation of ara-GTP accumulation in AML cells by fludarabine. Leukemia cells were obtained prior to therapy (▲) or 4 h after the end of fludarabine infusion (A) and incubated either with indicated concentration of ara-G for 3 h (▲) or with 100 μM ara-G for up to 4 h (B). The cells were extracted with perchloric acid and analyzed as described in “Patients and Methods.”

### DISCUSSION

As is the case for other arabinosyl nucleoside analogues such as ara-C (29, 30), the cytotoxicity of ara-G is thought to be due to cellular accumulation of the analogue triphosphate ara-GTP, its incorporation into DNA, and the consequent inhibition of DNA synthesis. In vitro work using human leukemia cell lines (6, 8, 9, 31) and primary leukemia cells obtained from patients (7) demonstrated that the differential accumulation of ara-GTP, the active metabolite, might be the mechanism for the selective sensitivity of T cells to this drug. Differential accumulation of intracellular ara-GTP in T-ALL versus other leukemia cells (AML, T-CLL, or B-lineage) was also observed during a Phase I trial with GW506U (17). Furthermore, it was demonstrated that patients who responded to this therapy (complete or partial response) achieved a significantly greater concentration of ara-GTP (median, 150 μM) in the circulating cells than patients who did not respond (median, 50 μM). These differences suggested that clinical benefits may be achieved in non-T-ALL patients if concentrations of ara-GTP could be increased

![ara-GTP accumulation](image-url)
and cladribine, are high $K_m$ substrates for dCK (23, 34); hence, a concentration greater than 20 $\mu$M is expected to further increase the rate of triphosphate accumulation. However, such concentrations of these analogues are not achievable during therapy due to toxicity. With respect to phosphorylation kinetics, ara-G shares characteristics of both pyrimidine and purine nucleoside analogues. At low concentrations (similar to pyrimidine analogues), the dGK activity may phosphorylate ara-G with a $K_m$ that is similar to the natural substrate ($K_m$, 7–65 $\mu$M; Refs. 5 and 12). At high concentrations, ara-G may be phosphorylated by dCK, albeit at a low affinity ($K_m$, <1 $\mu$M deoxycytidine; >100 $\mu$M ara-G; Refs. 5 and 23) like other purine nucleoside analogues. However, unlike fludarabine and cladribine, an ara-G concentration of >100 $\mu$M was achieved during a recent Phase I trial of GW506U (17).

The unique phosphorylation characteristics of ara-G predict that higher ara-G plasma concentrations would support a greater rate of ara-GTP synthesis. Accordingly, a Phase I protocol has been designed to administer GW506U at increasingly higher dose rates on a day 1, 3, and 5 schedule. Because the median half-life of ara-GTP was >24 h in circulating leukemia cells (17), this prolonged retention of ara-GTP provides an additional rationale to administer the drug on an alternate-day schedule.

The final approach used in the present work was a biochemical modulation strategy based on knowledge that the activities of dGK and dCK are feedback inhibited by dGTP and dCTP, respectively (10, 25, 26, 27, 32). The major metabolic pathway for the generation of these deoxynucleotides is catalyzed by ribonucleotide reductase. Hence, inhibition of this enzyme and lowering the cellular concentrations of these regulatory deoxynucleotides could activate the kinases that are involved in ara-G phosphorylation. Such a strategy using fludarabine or cladribine as an inhibitor of ribonucleotide reductase has been successful in increasing the dCK-dependent phosphorylation of ara-C in cell lines and primary leukemia cells in vitro and during therapy (28, 35–37). Furthermore, clinical combination of fludarabine and ara-C for patients with AML resulted in improved clinical response without untoward toxicity (38). Augmentation of ara-GTP accumulation in both B-CLL and AML cells (Figs. 3 and 4) provide rationales for similar therapeutic combinations for GW506U. To this end, a Phase I protocol has been designed to administer the standard dose of fludarabine 4 h prior to GW506U on days 1, 3, and 5.

In conclusion, we have demonstrated three approaches that augment the accumulation of ara-GTP in target cells and suggest new designs of protocols for clinical implementation. Clinically, we are using the first strategy in a protocol by infusing GW506U for 4 h, which may result in longer duration of plasma ara-G concentrations that sustain ara-GTP synthesis, and may, therefore, result in increased accumulation of ara-GTP in the target cells. The second tactic is being tested in the clinic by administration of GW506U on a day 1, 3, and 5 schedule. An every-other-day treatment protocol may allow higher doses to be administered each day, which may result in increased levels of intracellular ara-GTP. The third approach uses biochemical modulation of dGK and dCK, the enzymes that limit the rate of phosphorylation of ara-G to its active metabolite, ara-GTP. Clinically, this rationale will be tested with a sequential combination of fludarabine and GW506U, which should decrease regulatory deoxynucleotide triphosphates and activate ara-G phosphorylation. These three strategies are based on the mechanistic rationales, validated by in vitro experiments with primary human leukemia cells, that are now being translated into therapeutic applications.

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Pharmacological and biochemical strategies to increase the accumulation of arabinofuranosylguanine triphosphate in primary human leukemia cells.

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