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ORIGINAL ARTICLE

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Biochemical modulation of cytarabine triphosphate by clofarabine

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Abstract *Purpose*: Clofarabine has proven to be effective in the treatment of adult and pediatric acute myelogenous leukemia (AML). To investigate if clofarabine could be used with success in biochemical modulation strategies, we investigated the biochemical modulation of cytarabine triphosphate (ara-CTP) by clofarabine in a myeloid leukemia cell line and the effect of this combination on cytotoxicity. *Experimental design*: K562 cells were incubated with clofarabine and ara-C either sequentially or simultaneously to evaluate the combination effect on their phosphorylated metabolites. Clonogenic assays were used to determine the cytotoxicity of each agent alone and in combination. Deoxynucleotide analysis was performed to assess the effect of clofarabine on dNTPs. *Results*: Clofarabine added either simultaneously or in sequence increased ara-CTP accumulation. The maximal modulation of ara-CTP accumulation occurred with 1 μ*M* clofarabine. This level was achieved at the maximum tolerated dose for adult and pediatric patients with AML. With 10 μ*M* ara-C alone, 86 μ*M* ara-CTP had accumulated after 3 h. The optimal sequence for the drug combination, i.e., clofarabine followed 4 h later by ara-C, resulted in 248 μ*M* ara-CTP at 3 h. Clofarabine accumulated maximally in the monophosphate form. Preincubation with ara-C did not affect the triphosphate form, but it lowered clofarabine monophosphate. Clofarabine resulted in the intracellular decrease of dATP and dGTP levels. Clonogenic assays revealed that the combination of clofarabine and ara-C produced synergistic killing of myeloid leukemia cells. *Conclusions*: These findings demonstrate that combination of clofarabine followed by ara-C results in a biochemical modulation of ara-CTP and synergistic cell kill. These studies provide a compelling rationale for clinical trials using this combination regimen for adult and pediatric patients with AML.

Keywords Cytarabine triphosphate · Clofarabine · Biochemical modulation

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Introduction

Cytarabine (ara-C) is one of the most effective chemotherapeutic agents for the treatment of adult and pediatric acute myelogenous leukemia (AML) [35]. Ara-C, however, is a prodrug and needs to be converted to its active form, ara-C 5′-triphosphate (ara-CTP), by a series of intracellular enzyme-dependent phosphorylation steps. The first of these is rate limiting and is mediated by deoxycytidine (dCyd) kinase. The activity of dCyd kinase is inhibited by the dCyd triphosphate (dCTP) through a feedback mechanism [27]. Clinical and pharmacokinetic investigations in relapsed acute leukemias have demonstrated a strong relationship between intracellular ara-CTP and response to cytarabine [6, 25]. As a result, scientific efforts have focused on enhancing the activity of dCyd kinase to biochemically modulate ara-CTP accumulation.

Biochemical modulation of ara-C by nucleoside analogs, such as fludarabine and cladribine, has been shown to be feasible in studies of adult leukemias and pediatric AML [2, 4, 9, 11, 12]. Although as single

agents these purine nucleoside analogs are not effective for the treatment of adult acute leukemias, they have been successfully used in combination with ara-C. This is based on the observation that purine nucleoside analogs stimulate the accumulation of ara-CTP either by a direct effect on dCyd kinase or indirectly by inhibition of ribonucleotide reductase, an enzyme responsible for de novo synthesis of deoxynucleotides. This action on ribonucleotide reductase results in a decline in the intracellular dCTP pool, and therefore decreasing feedback inhibition of dCyd kinase. Independent of the feedback regulation of dCyd kinase, the activity and level of this enzyme are also increased by DNA-damaging agents and nucleoside analogs [38]. These findings were based on the results of in vitro investigations in leukemic cell lines [9, 36, 38], observations in primary leukemic and normal cells [10, 38], and in vivo results in adult patients with chronic lymphocytic leukemia (CLL) [11, 14] and adult [12] and pediatric patients with AML [2, 4].

Clofarabine, a new deoxyadenosine analog, was developed with the intent of combining the favorable mechanistic properties of fludarabine and cladribine [28]. Similar to these analogs, clofarabine is resistant to deamination by adenosine deaminase. In contrast to these agents, in clofarabine, the addition of fluorine in the arabinose configuration decreases susceptibility to enzymatic phosphorolysis by *Escherichia coli* purine nucleoside phosphorylase and improves the acid stability of the compound [3]. Mechanistically, this analog combines the potent inhibitory activity on ribonucleotide reductase and DNA synthesis after incorporation into DNA [29–31, 40, 41].

Fludarabine and cladribine are effective agents alone and in combination for indolent leukemias but are without significant activity in acute leukemias [22, 32]. In contrast, phase I studies with single-agent clofarabine have demonstrated its efficacy at the maximum tolerated dose (MTD) (40 mg/m^2 per day for 5 days) in adult acute leukemias with the dose-limiting toxicity being reversible hepatotoxicity [21]. Phase II studies have revealed an overall response rate of 48% at the MTD in adult patients with a diagnosis of AML, MDS, ALL, or CML-BP. In addition, these phase I and II studies have established that a favorable pharmacokinetic profile of clofarabine triphosphate is associated with clinical responses observed with this agent [15, 20]. The efficacy of clofarabine has also been tested in pediatric acute leukemias with responses observed in both AML and ALL [18]. This clinical activity of single-agent clofarabine in both adult and pediatric acute leukemia provided a compelling rationale to develop a biochemical modulation strategy using this purine nucleoside analog in combination with ara-C. The present study was conducted in a myeloid leukemia cell line model system to test biochemical modulation of ara-CTP by clofarabine and the effect of this combination on cytotoxicity.

Materials and methods

Cell line

The K562 cell line, derived from a patient with chronic myelogenous leukemia in blastic phase, was obtained from American Type Culture Collection (Rockville, Md.), and used throughout the study. The cells were maintained in suspension culture in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in exponential growth phase. Cell number and cell mean volume were determined before, during and after the incubation using a Coulter counter (Coulter Electronics, Hialeah, Fl.). The doubling time was approximately 24 h. Cell cultures were periodically certified free of mycoplasma using a commercially available kit and following the manufacturer's recommendations (Gen Probe, San Diego, Calif.).

Chemicals

Ara-C was obtained from Sigma Chemical Co. (St Louis, Mo.). Clofarabine was supplied by Dr. John Secrist III (Southern Research Institute, Birmingham, Ala.). For high-pressure liquid chromatography (HPLC) purposes, ara-CTP was purchased from Sigma Chemical Co. For cellular pharmacokinetics as a HPLC standard, clofarabine 5′-triphosphate (Cl-F-ara-ATP) was synthesized by Sierra BioResearch (Tucson, Ariz.).

Accumulation of phosphorylated ara-C metabolites

Exponentially growing cells were incubated with 1.0 μ*M* clofarabine and 10 μ*M* [^{3}H]ara-C for the times and sequence indicated. Cells were washed twice with phosphate buffer. Cells were resuspended in fresh medium and aliquots were taken at 1, 3, and 5 h. Nucleotides were extracted using $HClO_4$ for each sample. Ara-CMP, ara-CDP, and ara-CTP were separated from other nucleotides by HPLC using a Paritsil 10 SAX (Whatman, Clifton, N.J.) anion exchange column as described previously [33].

The identity of the nucleoside analog metabolites was confirmed by their coelution with authentic ara-C metabolites, by their resistance to periodate oxidation, and by their ratio of absorbance (ara-CTP, 280 nm/254 nm = 3.11). The quantitation of ara-CTP was done at 280 nm and clofarabine triphosphate at 257 nm. The radioactivity associated with the respective nucleotides was measured with a radioactive flow detector (Model A250; Packard instrument Co., Meridien, Ct.).

Accumulation of phosphorylated clofarabine metabolites

Exponentially growing cells were incubated with 1.0 μ*M* [^{3}H]clofarabine and 10 μ*M* ara-C for the times and sequence indicated. Cells were washed twice with phosphate RPMI supplemented with 10% inactivated fetal bovine serum. Cells were resuspended in fresh medium and aliquots were taken at 1, 2, 3, and 4 h. Nucleotides were extracted using $HCLO_4$ for each sample. Clofarabine monophosphate, diphosphate, and triphosphate were separated from other nucleotides by HPLC as described previously [40].

Clonogenicity

K562 cells were incubated without drug, with 1 μ*M* clofarabine, with 10 μ*M* ara-C, or combination of both analogs. The cells were washed twice with drug-free warm medium. For each condition 300–3000 cells were mixed with 0.3% soft agar in DMEM supplemented with 30% fetal bovine serum (prewarmed to 37°C) then incubated in 35×10 mm tissue culture dishes for 10 days (humidified atmosphere containing 5% CO_2 at 37°C). At the end of the incubation period, colonies of more than 30 cells were scored with the aid of an inverted microscope. The cytotoxic effect of the drug was expressed as a percentage of surviving cells relative to the untreated control cultures.

Effect of clofarabine on dNTP pools

K562 cells were treated with various concentrations of clofarabine as indicated and nucleotides were extracted using 60% methanol and stored at −20°C. The DNA polymerase assay as modified by Sherman and Fyfe [37] was used to quantitate dATP, dCTP, dGTP, and dTTP pools in the cell extracts. The Klenow fragment of DNA polymerase lacking $3' \rightarrow 5'$ exonuclease activity (US Biochemical Corporation, Cleveland, Ohio) was used to start a reaction in a mixture that contained 100 μ*M* HEPES buffer, pH 7.3, 10 μ*M* $MgCl_2$, 7.5 μg BSA, and synthetic oligonucleotides of defined sequences as templates annealed to a primer, [^{3}H]dTTP and either the standard dATP, or [^{3}H]dATP and the standards for dGTP, dCTP, dTTP, or the K562 cell extracts. Reactants were incubated for 1 h, and applied to filter disks; after they were washed, the radioactivity on the disks was determined by liquid scintillation counting and compared with that in the standard dNTP samples.

Results

Optimal clofarabine concentration for ara-CTP accumulation

To determine the optimal concentration necessary for maximal ara-CTP accumulation, K562 cells were incubated without or with 0.03, 0.1, 1.0, and 3 μ*M* of clofarabine for 4 h and then 10 μ*M* of ara-C was added. Ara-CTP accumulation was measured 3 h later. Without clofarabine, the accumulation of ara-CTP was consistent with previously published data at a level of 110 ± 32 μ*M* [9]. Preincubation with clofarabine at 0.03, 0.1, 1.0, and 3 μ*M* resulted in 158 ± 49, 234 ± 71, 448 ± 41, and 444 ± 25 μ*M*, of ara-CTP, respectively (Fig. 1). These data suggest an increase in ara-CTP accumulation, which was dependent on the dose of clofarabine. However, the maximal modulation of ara-CTP was achieved at 1 μ*M* of clofarabine. Such concentrations (1–2 μ*M*) of clofarabine are achieved in plasma at the MTD in adults (40 mg/m^2 per day) [15, 20, 21] or (52 mg/m^2 day) in pediatric patients [18]. Hence, additional experiments were performed using 1 μ*M* of clofarabine.

Effect of schedule on ara-CTP accumulation

To identify the effect of schedule of 1 μ*M* clofarabine on ara-CTP accumulation, cells were incubated under four different conditions. The first culture was treated with 10 μ*M* ara-C alone, while in the second culture the cells were simultaneously incubated with clofarabine and ara-C. The next two cultures were pretreated for 4 h with clofarabine and cells were either washed or not washed free of clofarabine prior to the addition of ara-C (Fig. 2). Compared to ara-C alone, there was an increase in ara-CTP accumulation following the addition of clofarabine. For example, under these conditions, the intracellular concentrations of ara-CTP at 3 h were 86 ± 12, 131 ± 36, 163 ± 24 μ*M*, or 248 ± 66 μ*M*, respectively. Therefore, 1 μ*M* of clofarabine followed 4 h later by 10 μ*M* of ara-C had the greater effect on accumulation of ara-CTP and continued presence of clofarabine

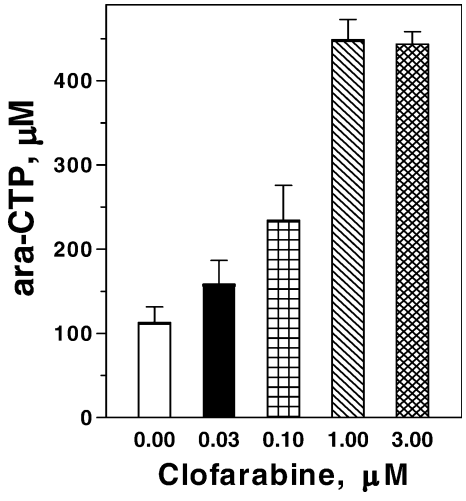

Fig. 1 Optimal concentration of clofarabine for modulation of ara-CTP accumulation. K562 cells were incubated without or with 0.03, 0.1, 1.0, and 3.0 μ*M* clofarabine for 4 h. To the same cultures, 10 μ*M* ara-C was added and accumulation of ara-CTP was measured after 3 h as described in "Materials and methods"

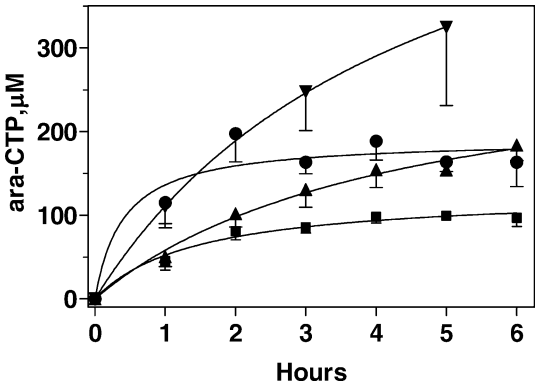

Fig. 2 The effect of schedule of clofarabine on ara-CTP accumulation. Cells were incubated under four different conditions: ara-C alone (*filled squares*), simultaneous addition of clofarabine + ara-C (*up filled triangles*), clofarabine for 4 h followed by washing with phosphate buffer and then the addition of ara-C (*filled circles*), and clofarabine for 4 h, followed by addition of ara-C without any washing steps (*down filled triangles*). The concentration of clofarabine was 1 μ*M* and the concentration of ara-C was 10 μ*M*

had the highest effect. These results suggest that sequential rather than simultaneous addition of the two agents may be of more benefit for maximal ara-CTP accumulation.

Effect of clofarabine on the rate of ara-CTP accumulation

In the previous experiment (Fig. 2), the rate of accumulation of ara-CTP was linear only up to 2 h. Therefore, we wanted to further define the influence of clofarabine on the rate of ara-CTP accumulation. For this, K562 cells were incubated with ara-C alone, clofarabine + ara-C, or clofarabine followed 4 h later by ara-C (Fig. 3). Cell aliquots were removed every 20 min and extracted with perchloric acid. The rate of ara-CTP accumulation was linear under all three conditions, and the rate with ara-C alone was 18 μ*M*/h. In contrast, the

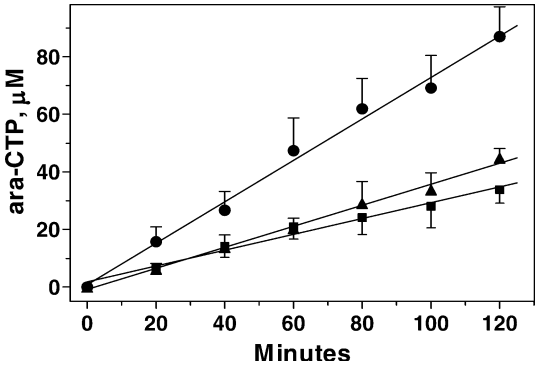

Fig. 3 The effect of clofarabine on the rate of ara-CTP accumulation. Cells were incubated under three conditions: ara-C alone (*filled squares*), clofarabine + ara-C (*filled triangles*) and clofarabine followed 4 h later by ara-C (*filled circles*). For the last of these conditions, cells were not washed with phosphate buffer following incubation with clofarabine. Cell aliquots were removed every 20 min and extracted with perchloric acid

rates of ara-CTP accumulation were 21 μ*M*/h and 44 μ*M*/h with the simultaneous and sequential addition of clofarabine and ara-C, respectively. Therefore, the rate of ara-CTP accumulation was maximal with the sequential addition of clofarabine followed 4 h later by ara-C.

Effect of clofarabine on phosphorylated ara-C metabolites

To determine if the modulatory effect of clofarabine was evident on the monophosphate, diphosphate, and triphosphate metabolites of ara-C, cells were incubated for 1, 3, and 5 h with [^{3}H] ara-C alone, [^{3}H] ara-C + clofarabine, or [^{3}H] ara-C after clofarabine (Fig. 4). Compared to ara-C alone, sequential combination of clofarabine and ara-C resulted in the greatest increase in monophosphorylated, diphosphorylated, and triphosphorylated metabolites of ara-C, which was time dependent with maximal modulation at 5 h. At this time point, the monophosphate, diphosphate, and triphosphate, respectively, were 2.2, 3.4, and 3.4 times greater than those of ara-C alone. Among all phosphorylated metabolites, ara-CTP was the maximum (>85%) and increased proportionally with clofarabine. The paucity of monophosphate and diphosphate of ara-C relative to ara-CTP indicates that phosphorylation of ara-C by dCyd kinase is the only rate limiting step in triphosphate synthesis.

Effect of ara-C on phosphorylated clofarabine metabolites

To determine the effect of ara-C on clofarabine and its metabolites, exponentially growing K562 cells were

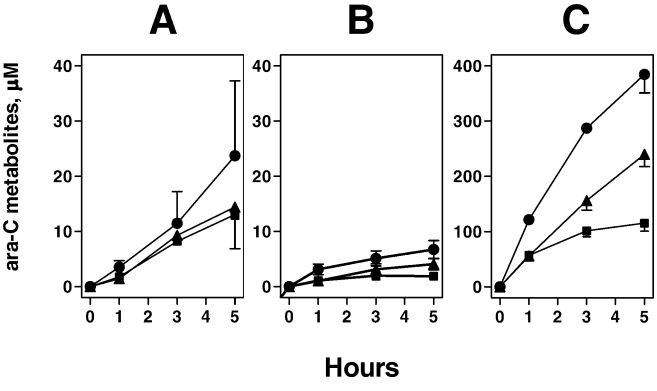

Fig. 4 The effect of clofarabine on phosphorylated ara-C metabolites. Cells were incubated under three different conditions: [^{3}H] ara-C alone (*filled squares*), clofarabine + [^{3}H]ara-C (*filled triangles*), and clofarabine followed by [^{3}H]ara-C (*filled circles*). For the last of these conditions, cells were not washed with phosphate buffer following incubation with clofarabine. Cell aliquots were extracted with perchloric acid at 0, 1, 3, and 5 h and accumulation of (**A**) ara-CMP, (**B**) ara-CDP, and (**C**) ara-CTP was measured using HPLC

treated with 1 μ*M* of clofarabine alone, or 1 μ*M* of [^{3}H]clofarabine + ara-C, or ara-C followed by [^{3}H]clofarabine. Nucleotides were extracted and separated by HPLC. Among all phosphorylated clofarabine metabolites, the monophosphate accumulation was the greatest; at 4 h it was 4.9 times greater than the level of clofarabine triphosphate (compare Fig. 5a with Fig. 5c). Ara-C incubation did not affect clofarabine triphosphate accumulation, but did lower clofarabine monophosphate accumulation. Sequential addition of clofarabine to ara-C lowered the monophosphate accumulation by 19%, whereas simultaneous addition of the two drugs lowered monophosphate accumulation by 86%.

Effect of clofarabine and ara-C on clonogenicity

Clonogenic assays were used to quantitate the loss of viability of K562 cells after treatment under the following conditions: no drug treatment (control), treatment with 10 μ*M* ara-C alone, treatment with 1 μ*M* clofarabine alone, treatment of clofarabine + ara-C, and treatment with clofarabine followed 4 h later by ara-C (Fig. 6). Compared to untreated (control) cells, treatment with 10 μ*M* ara-C or 1 μ*M* clofarabine alone resulted in losses of cell viability of 19% and 28%, respectively. However, when cells were exposed to the two combinations of the drugs, the losses of cell viability became 88% and 82%, respectively. Previous studies have demonstrated that clofarabine is a potent cytotoxic agent in cell lines such as CEM, K562, Hep2, and murine leukemia L1210 [28, 29, 40]. It has been postulated that this could be due to factors such as its resistance to deamination and enzymatic phosphorolysis, as well as its relatively slow rate of elimination from cells [40, 41]. Our findings demonstrate, however, that compared with treatment with the single agents, the combination of ara-C and clofarabine was synergistic for cell death.

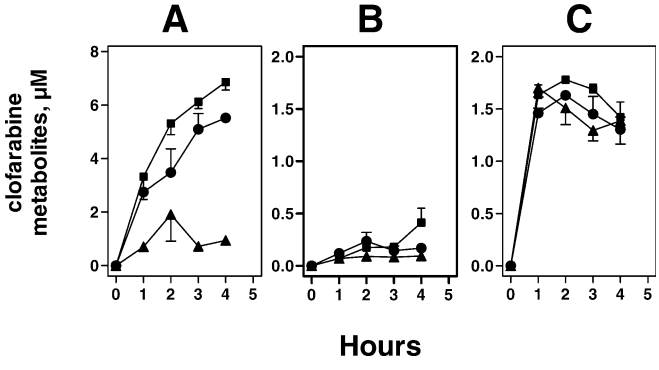

Fig. 5 The effect of ara-C on phosphorylated clofarabine metabolites. Cells were incubated under three different conditions: [^{3}H]clofarabine alone (*filled squares*), ara-C + [^{3}H]clofarabine (*filled triangles*), and ara-C followed by [^{3}H]clofarabine (*filled circles*). Cells were not washed with phosphate buffer following incubation with ara-C. Cell aliquots were extracted with perchloric acid at 0, 1, 2, 3, and 4 h and accumulation of (**A**) clofarabine monophosphate, (**B**) clofarabine diphosphate, and (**C**) clofarabine triphosphate was measured using HPLC

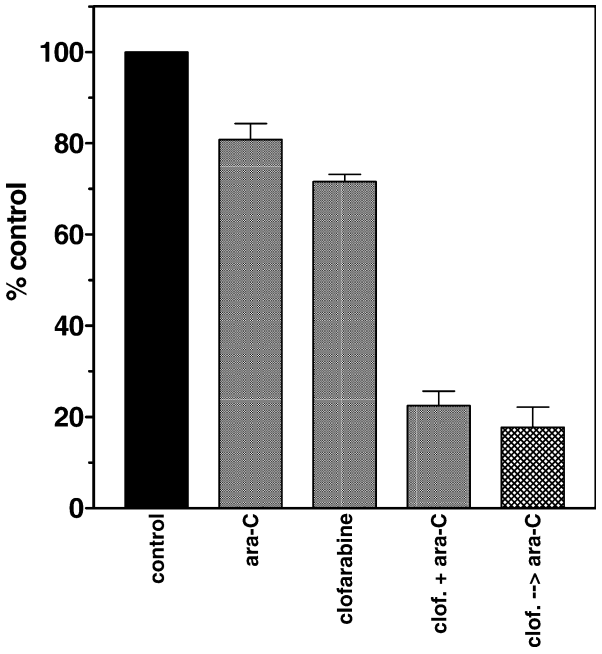

Fig. 6 Clonogenicity of K562 cells in response to treatment with clofarabine. Cells were incubated with ara-C alone, clofarabine alone, or with the combinations of the two drugs. Cells were washed and clonogenicity assays were performed. Maximal inhibition of survival occurred with clofarabine followed 4 h later by ara-C

Effect of clofarabine on cellular deoxynucleotide concentration

To determine the effect of clofarabine on ribonucleotide reductase in K562 cells, the cellular concentrations of deoxynucleotides were quantitated in untreated cells and cells treated with various concentrations of clofarabine as presented in Table 1. The values listed in Table 1 are

Table 1 The effect of clofarabine on dNTP pools in K562 cells. Deoxynucleotides were quantitated by DNA polymerase assay as described in "Materials and methods." The levels of dNTPs in control cells were: dATP 13.8 ± 1.2 μ*M*, dCTP 7.6 ± 0.6 μ*M*, dGTP 4.7 ± 0.4 μ*M*, and dTTP 34.8 ± 4.5 μ*M*. Cells were incubated for 4 h with 0.03, 0.1, 1, and 3 μ*M* clofarabine and the levels of the deoxynucleotides were determined. The control levels were: dATP 13.8 ± 1.2 μ*M*, dCTP 7.6 ± 0.6 μ*M*, dGTP 4.7 ± 0.4 μ*M*, and dTTP 34.8 ± 4.5 μ*M*. Clofarabine had the greatest effect on dATP levels at a concentration of 1.0 μ*M*. The values presented are percent of control culture

	dATP	dCTP	dGTP	TTP
Control	100	100	100	100
0.03 μ*M* clofarabine	49	104	100	78
0.1 μ*M* clofarabine	22	110	83	94
1.0 μ*M* clofarabine	15	116	47	117
3.0 μ*M* clofarabine	16	110	40	113

percentages in relation to control cells and are the means from three different experiments. The cellular concentrations of dATP and dGTP were significantly affected by clofarabine treatment. Interestingly, the level of dCTP was not reduced. When these experiments were done using dialyzed serum to remove trace amounts of deoxynucleosides from the medium, the results were similar (data not shown).

Discussion

Deoxyadenosine analogs have proven to be important in the treatment of adult and pediatric leukemias. As single agents, fludarabine and cladribine have shown efficacy in the treatment of CLL and indolent lymphomas, and hairy cell and pediatric acute leukemias [22, 24, 32, 34]. Both have been used with ara-C in biochemical modulation strategies [2, 4, 11, 26, 39] and in preparative regimens for bone marrow transplantation [8].

As a biochemical modulator of ara-CTP, clofarabine needs to be tested as it may provide advantages over other deoxyadenosine analogs. First, with the addition of fluorine to the arabinose configuration, clofarabine possesses metabolic advantages over fludarabine and cladribine by being resistant to bacterial enzymatic and acidic phosphorolytic cleavage [3]. Second, this feature makes clofarabine a suitable oral drug [3]. Third, clofarabine possesses biochemical and pharmacokinetic advantages over fludarabine and cladribine. For example, pharmacokinetic studies of clofarabine as a single agent in the treatment of adult and pediatric acute leukemias have demonstrated a MTD of 40 mg/m^2 per day and 52 mg/m^2 per day, respectively [15, 18, 20, 21]. At the end of infusion at these doses, a median plasma level of 1–2 μM clofarabine is achieved. This plasma concentration is significant, as studies have demonstrated that clofarabine is an efficient substrate for the rate-limiting enzyme, dCyd kinase, with a K_m of 14 μM [31]. Therefore, the MTD of clofarabine in the treatment of acute leukemias allows adequate plasma concentrations as well as efficient interaction with dCyd kinase in the biochemical modulation strategy. In contrast, the MTD of fludarabine is 30 mg/m^2 per day and at this dose the plasma concentration of the drug is about 3 μM. However, fludarabine is not an efficient substrate for dCyd kinase, with a K_m of 1600 μM [31]. Cladribine is an efficient substrate for dCyd kinase with a K_m of 5 μM [31]. However, at the MTD of 10 mg/m^2 per day, the maximum median plasma concentration of cladribine is 0.1 μM [1]. Therefore, clofarabine has advantages over both fludarabine and cladribine because of its plasma concentration at the MTD and its affinity for phosphorylation by dCyd kinase.

Fourth, because the modulation of ara-CTP accumulation is dependent on the concentration of analog triphosphates in the cell rather than the plasma level of the drug, comparison of fludarabine, cladribine, and clofarabine is necessary. Unlike fludarabine and other conventional analogs, both cladribine and clofarabine monophosphates seem to be converted to their diphosphate form with difficulty. As a result, at the MTD, the levels of cladribine and clofarabine triphosphates are much lower [15, 20] than that of fludarabine triphosphate [5, 13, 23] during therapy. However, studies demonstrate that clofarabine triphosphate has longer retention times in blasts of patients with acute leukemias than fludarabine or cladribine triphosphates [1, 13, 20].

Fifth, its toxicity profile indicates that clofarabine is suitable for combination with ara-C. With single-agent ara-C, neurotoxicity including central nervous system associated effects and pulmonary toxicity are dose-limiting [17, 19]. In contrast, single-agent clofarabine toxicity is hepatic in nature [21]. These non-overlapping toxicity profiles of these two agents may allow full-dose administration of each drug without untoward effects as observed with the current combination regimen [7].

Finally, and most importantly, clofarabine has a clinical advantage over fludarabine and cladribine for adult and pediatric acute leukemias. The clinical response in adult populations at the MTD of clofarabine was 48% [20]. Similarly, even in phase I study, a 25% response rate was achieved in pediatric leukemias [18].

Taken together, these pharmacokinetic, biochemical, and clinical observations provide a compelling rationale for testing the clofarabine and ara-C combination in adult and pediatric acute leukemias. In clonogenic assays, compared with single agents, in the K562 model system, both simultaneous and sequential combinations of clofarabine and ara-C showed synergistic cytotoxicity (Fig. 6). The challenge is to determine which of these schedules would translate into improved clinical responses.

The first approach would be to combine both agents simultaneously; for example, continuous infusion of clofarabine at the MTD combined simultaneously with a continuous infusion of ara-C at a dose of 500–1000 mg/m^2 per day over 5 days. This schedule results in 1 μM ara-C in plasma, a concentration that has been shown to be cytotoxic to cells [16]. Continuous infusion of clofarabine at 40 mg/m^2 per day or 52 mg/m^2 per day would result in plasma concentrations in the nanomolar range. As ara-C is a better substrate for dCyd kinase, low plasma concentrations of clofarabine would not be optimally phosphorylated in the presence of 1 μM ara-C. In addition, the continuous infusion of clofarabine is not necessary as the half-life of clofarabine triphosphate is >24 h in leukemic blasts. Therefore, pharmacologically, combining the two drugs as a continuous infusion does not appear to be an optimal strategy.

A second option for the treatment schedule of this biochemical modulation strategy is to combine daily bolus infusions of clofarabine with continuous infusion of ara-C for 5 days. The rationale for this schedule has been tested with other deoxyadenosine analogs and ara-C. In vitro studies have demonstrated the capacity of clinically attainable concentrations of fludarabine

triphosphate to enhance the formation of ara-CTP at concentrations of ara-C that are achieved during a continuous infusion schedule [36]. This strategy has been tested clinically using fludarabine and continuous infusion ara-C in a phase I trial in pediatric patients with relapsed acute leukemias. The combination therapy successfully eradicated bone marrow disease in 16 of 27 patients (59%). A randomized study compared two schedules of ara-C, continuous infusion versus bolus, in combination with cladribine in pediatric patients with newly diagnosed primary AML [4]. Although ara-CTP accumulation was similar in both treatment arms, the continuous ara-C arm had a significantly better response on day 15 after the start of therapy (63%) when compared with the arm utilizing bolus infusions of ara-C (32%) [4]. Taken together, these data strongly suggest combination of continuous ara-C with bolus clofarabine.

A third schedule for this biochemical modulation strategy could be to combine a short infusion of clofarabine at the MTD followed 4 h later with a 1-h infusion of an intermediate-dose rate of ara-C (500 mg/m^2 per hour) which allows maximal ara-CTP accumulation. By adding ara-C 4 h after the clofarabine, there is decreased competition for dCyd kinase and, therefore maximal phosphorylation of both agents. Additionally, clofarabine triphosphate achieves a peak 4 h after the start of clofarabine infusion. Hence, maximal modulation of ara-CTP is expected at this time point. Because clofarabine triphosphate has a long half-life in leukemic blasts, it is not necessary for clofarabine to be administered as a continuous infusion. Taken in concert, sequential bolus infusion of each drug is also a viable option for combination of these agents.

In conclusion, our in vitro data in the K562 cell line suggests that combination therapy with clofarabine and ara-C would be effective in the treatment of acute leukemias. Currently, we are testing such an approach in adult acute leukemia patients [7].

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