A phase 1 clinical-laboratory study of clofarabine followed by cyclophosphamide for adults with refractory acute leukemias

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Clofarabine has shown impressive response rates in patients with acute leukemias. In vitro investigations with clofarabine in combination with cyclophosphamide in primary cells have demonstrated synergistic cytotoxicity and inhibition of DNA repair. Based on these clinical and laboratory observations, we designed a mechanism-based combination protocol with clofarabine and cyclophosphamide for patients with relapsed acute leukemias. Eighteen patients were treated with cyclophosphamide (200 mg/m²) alone on day 0 and with clofarabine plus cyclophosphamide on day 1. Clinical responses, toxicity, DNA damage measured as H2AX phosphorylation, and accumulation of clofarabine triphosphate (TP) were analyzed. At dose level 1 (20 mg/m² clofarabine + cyclophosphamide, 6 patients) and dose level 0 (10 mg/m² clofarabine + cyclophosphamide, 12 patients) overall response rates were 50% and 30%, respectively, with responses in 4 (67%) of 6 patients with refractory acute lymphoblastic leukemia. Dose-limiting toxicity occurred at dose level 1 with prolonged marrow aplasia. Four (22%) patients died from prolonged aplasia (1), fungal pneumonia (1), or multiorgan failure (2). In 12 of 13 patient samples, increased DNA damage (γH2AX) was observed with clofarabine and cyclophosphamide compared with cyclophosphamide alone. In conclusion, pharmacodynamic end points along with clinical results suggest usefulness of this combination strategy, whereas toxicity data suggest reduction in chemotherapeutic intensity. This clinical trial is registered with the National Cancer Institute’s PDQ at www.clinicaltrials.gov as no. JHOC-J0561. (Blood. 2007;110:1762-1769)

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Introduction

Refractory and relapsed acute leukemias are characterized by an interplay of cellular mechanisms that shift the balance between cell survival and death pathways toward prolonged survival and, in turn, net drug resistance. The clinical outlook for such patients is very poor, with complete remissions (CRs) being achieved in 30% or fewer and a 1-year disease-free survival (DFS) of less than 10% with the use of current antileukemic agents. As a result, there is no standard of care for these diseases at the present time. In order to improve our results, one goal of any new therapeutic approach must be the ability to overcome drug resistance and promote drug-induced cell death. This may be achieved by mechanism-based combination chemotherapy.

Clofarabine is a novel deoxyadenosine analog that is resistant to deamination by adenosine deaminase and phosphorolytic cleavage by bacterial purine nucleoside phosphorylase. Clofarabine has clinical activity as a single agent and in combination with cytosine arabinoside (ara-C) against refractory and relapsed pediatric acute lymphoblastic leukemia (ALL) and against relapsed and poor-risk, newly diagnosed acute myelogenous leukemia (AML) in adults, including those age 50 years and older. Clofarabine exerts its cytotoxicity through multiple mechanisms of action, with a major effect on ribonucleotide reductase (RR) inhibition and incorporation into DNA followed by inhibition of DNA polymerases. Additional effects on mitochondrial membrane polarization and disruption with resultant cytochrome c release and apoptosis induction have been reported.

Ribonucleotide reductase converts ribonucleotides into deoxyribonucleotides and is therefore a pivotal enzyme in the processes of DNA synthesis and repair of DNA damage. Inhibition of RR depletes the intracellular pools of deoxyribonucleotides and their triphosphorylated forms (dNTPs). The depletion enhances the intracellular accumulation of nucleoside analogs and the incorporation of triphosphorylated nucleoside analogs into DNA, thereby heightening the cytotoxic effects of those agents.

Cyclophosphamide (CY; Cytoxan) is an alkylating agent that is effectively used for treatment of leukemias. A primary lesion of CY damage is DNA interstrand cross-links. These cross-links have been seen to be rapidly repaired in chronic lymphocytic leukemia (CLL) lymphocytes after in vitro exposure to activated CY. However, pretreatment with clofarabine impedes completion of the repair of DNA strand breaks, with resultant increase in apoptosis. This effect may be due to inhibition of both RR and DNA synthesis by clofarabine triphosphate (clofarabine-TP).

On the basis of these findings and rationale, we designed a phase 1 clinical-laboratory correlative trial to test the hypothesis that pretreatment with clofarabine would impede the full repair of CY-induced DNA strand breaks and would therefore augment CY-driven cytotoxicity in vivo and possibly improve clinical responses in patients with relapsed or refractory acute leukemias. We delivered the drug combination in a timed sequential fashion. To confirm that CY-induced DNA damage was enhanced by clofarabine pretreatment, we measured leukemia cell DNA

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damage directly and serially during treatment. In order to test for potential augmentation of CY-induced DNA damage by clofarabine, we compared DNA damage with CY alone and with CY following clofarabine pretreatment.

Patients, materials, and methods

Patient eligibility and selection

Between December 2005 and August 2006, 18 adults (age ≥ 18 years) were entered on study at the Johns Hopkins Sidney Kimmel Cancer Center. Patients were required to have confirmed diagnoses of relapsed and/or refractory AML, including AML arising from myelodysplasia (MDS) or myeloproliferative disorder (MPD); ALL, including Philadelphia chromosome–positive (Ph+) ALL; or chronic myelogenous leukemia (CML) in accelerated phase (AP) or blast crisis (BC) of either myeloid or lymphoid origin that was resistant to imatinib.

Other eligibility criteria included an Eastern Cooperative Oncology Group (ECOG) performance status of 2 or lower, serum creatinine level 176.8 μM (2.0 mg/dL) or lower, hepatic transaminase levels 5 × normal or lower, serum total bilirubin level 25.65 μM (1.5 mg/dL) or lower, negative pregnancy test within 48 hours prior to study, willingness to practice contraception, no more than 4 prior induction regimens (3 cytotoxic chemotherapy regimens), inability for established curative regimens including stem-cell transplantation (SCT), and ability to provide informed consent. Patients who were unresponsive to or had relapsed after allogeneic or autologous SCT were eligible. The interval from prior treatment was at least 3 weeks for myelosuppressive cytotoxic agents (6 weeks for mitomycin or nitrosoureas), 2 weeks for radiotherapy, and 1 week for either nonmyelosuppressive treatments or treatment with hematopoietic growth factors. Hydroxyurea (HU) and/or imatinib were permitted for control of blast counts prior to clofarabine-CY therapy but had to be discontinued at least 72 hours before initiation of clofarabine-CY. All patients provided written informed consent in accordance with the Declaration of Helsinki and Johns Hopkins Medical Institutional Review Boards and guidelines.

Treatment schema

This phase 1 study was designed to determine the maximal tolerated dose (MTD) and assess the toxicity of clofarabine followed by fractionated CY in adults with relapsed or refractory acute leukemias. An additional objective was to test the hypothesis that clofarabine would inhibit the repair process initiated by CY-induced DNA damage. Single-agent CY was administered by 2-hour intravenous infusion on day 0 of cycle 1. On days 1 to 3 and 8 to 10, clofarabine was administered by intravenous infusion 2 hours before each dose of CY. For dose level 1, the day 0 and 1 CY doses were 200 mg/m²/dose and the day 2, 3, and 8 to 10 doses were 400 mg/m²/dose; the clofarabine dose was 20 mg/m²/day on days 1 to 3 and 8 to 10. Dose escalations for both clofarabine and CY were planned, with step-wise increases in clofarabine dose up to 50 mg/m²/dose and CY up to 800 mg/m²/day. However, because of dose-limiting toxicity (DLT) at dose level 1, clofarabine dosage was de-escalated to 10 mg/m²/day (dose level 0).

Supportive care

Clofarabine-related infusional toxicities. Clofarabine administration has been associated with a systemic inflammatory response consisting of fever, respiratory distress, hypotension, capillary leak (pleural and pericardial effusions), and multiorgan failure.2,3,5,7 This constellation of symptoms has been most pronounced in the setting of drug-induced tumor lysis syndrome. To decrease the incidence and severity of this clofarabine-related cytokine-release syndrome, all patients were premedicated with acetaminophen 650 to 1000 mg orally 30 minutes prior to each dose of clofarabine to minimize the risk of fever and received prophylactic steroids (hydrocortisone 100 mg daily) on days 1, 2, and 3 prior to clofarabine dosing. Histamine (H1 and H2) blockade was also administered to patients with evidence of systemic inflammatory response syndrome or cytokine release manifested as tachypnea, tachycardia, fever, or hypotension temporally associated with clofarabine infusions.

Allopurinol. All patients without known allergy to allopurinol received allopurinol 300 mg/day on days 0 through 5 to prevent occurrence and complications of tumor lysis–induced hyperuricemia. Antibiotic prophylaxis. Patients received prophylaxis against Pneumocystis carinii pneumonia, Gram-negative gastrointestinal infections, and reactivation of herpes simplex. Antifungal prophylaxis or therapy with triazoles or echinocandins was not given during administration of clofarabine in order to avoid CYP450-related drug-drug interactions.

Response and toxicity evaluation

To assess response to therapy, bone marrow aspiration and biopsy were performed prior to treatment, on day 14, and at the time of hematologic recovery or when leukemia regrowth was suspected clinically. Hematologic recovery was defined as absolute neutrophil count (ANC) 500/μL or greater and a transfusion-independent platelet count 50 000/μL or greater. CR required a normal bone marrow aspirate with absence of identifiable leukemia, absence of peripheral blood blasts, ANC 1000/μL or greater, platelet count 100 000/μL or greater, and clearance of extramedullary disease including splenomegaly. Clearance of cytogenic abnormalities was not required for CR but was noted and described separately. Partial remission (PR) was defined as trilineage hematopoiesis in the marrow with normalization of peripheral blood counts but with 5% to 25% blasts in the marrow, at least 50% decrease in bone marrow blasts, or persistent splenomegaly. Response criteria for acute leukemias are consistent with those published by Cheson et al.16 Responses were determined following the first cycle of clofarabine-CY.

The National Cancer Institute (NCI) Common Toxicity Criteria (version 3.0) was the basis upon which all adverse events were described and graded, based on the treating physician’s assessment. DLT was based on toxicities incurred during the first treatment cycle. If 2 of 3 or 2 of 6 patients developed DLT, the MTD was defined as 1 dose level below that which the DLTs were observed. If 2 of 3 or 2 of 6 patients at dose level 1 experienced DLT, the dose of clofarabine was reduced to 10 mg/m²/day with the CY dose remaining constant. Myelosuppression was not considered in evaluating toxicity except where bone marrow hypoplasia persisted for at least 42 days with marrow cellularity 5% or lower and no evidence of leukemia. Myelosuppression-related DLT occurred when ANC did not return to 1 × 10⁹/L and platelet count did not return to 75 000/μL or 20% of baseline values (whichever was less) by day 42 of therapy.

Laboratory correlates

All studies of peripheral blood and bone marrow blast populations were conducted following blast enrichment by Ficoll density separation. For the 13 patients whose circulating blasts were analyzed longitudinally for DNA damage and apoptosis, the median percentage of blasts prior to Ficoll enrichment was 75% (range, 31%-96%) on day 0 prior to treatment and 68% (range, 29%-100%) on day 1 after CY alone and before beginning the clofarabine infusion. The minimal percentage of blasts following Ficoll enrichment was 70% for all specimens.

Measurement of DNA damage

Histone H2AX is phosphorylated in response to the presence of DNA double-strand breaks.15 As such, the presence and magnitude of phosphorylated H2AX (γH2AX) is an indication of persistent, unrepaired DNA damage.17,18 We measured DNA damage by flow cytometry of leukemia blasts stained with antibody to γH2AX. Peripheral blood blasts were sampled before treatment, at the completion of day 0 CY infusion, 2 hours after completion of day 0 CY infusion, and 2 hours after completion of the combination of clofarabine followed by CY on day 1. When feasible, bone marrow blasts were assayed for the presence and magnitude of γH2AX prior to treatment, on day 0 at the completion of the CY infusion, and on day 3 after finishing clofarabine followed by CY. In brief, peripheral blood and bone marrow blasts were enriched by Ficoll density gradient separation, fixed in 70% ethanol, and stored at 4°C until staining in TST buffer (TBS,
4% FBS, 0.1% Triton X-100). Cells were incubated in either a 1:500 dilution of antiphospho-kinase H2AX (Upstate Cell Signaling Solutions, Lake Placid, NY), a 1:500 dilution of mouse IgG1 isotype control (Southern Biotech, Birmingham, AL), or TST alone. After 2 hours incubation, cells were exposed for 1 hour to a 1:200 dilution of Alexa Fluor 488 goat antimouse IgG (H + L; Molecular Probes, Eugene, OR) with 5 μg/mL RNase, fixed in 2% formaldehyde in TBS, and stored at 4°C until flow-cytometric analysis. Cellular debris was gated out and nonspecific staining controls were used to mark the lower limit of the positive H2AX-staining region (SR). Control SR was subtracted from SR of samples to determine the percentage of cells positive for γH2AX (expressed as % γH2AX). Each flow analysis was performed on a standard sampling of 1 × 10^6 cells. The standard deviation for this method (triplicate sampling) is 0.3-fold or less.

**Measurement of apoptosis**

Apoptosis was estimated for leukemic blasts obtained serially as described in “Patients, Materials and methods, Measurement of DNA damage,” on days 0 and 1 from peripheral blood and/or bone marrow by calculating sub-2N DNA from cell-cycle analysis using propidium iodide (PI)–stained cell nuclei. Following fixation in 70% ethanol, blasts were permeabilized in 0.1% Triton X-100, treated with 5 μg/mL RNase and 50 μg/mL PL, and stored at 4°C until flow-cytometric analysis. The sub-2N fractions, quantified in ungated histograms, were defined as the samples’ apoptotic subpopulations.

**Intracellular pharmacology**

Peripheral blood cells were obtained on day 1 prior to clofarabine administration, at the end of the clofarabine infusion, at the end of the CY infusion, and 2 hours after completion of CY. Leukemic blasts were enriched by Ficoll density gradient separation and the cell number was determined by a Coulter counter (Coulter, Hialeah, FL). Cells were then processed for nucleotide extraction. In brief, nucleotides and clofarabine-TP were extracted from cells using standard procedures with HClO4 and separated on an anion-exchange Partisil-10 SAX column (Waters, Milford, MA) using high-performance liquid chromatography. The levels of triphosphates (clofarabine-TP and 4 NTPs) were calculated and expressed as the quantity of nucleotides contained in 1 × 10^6 cells. The lower limit of quantitation of the assay was approximately 1 pmol in an extract of 2 × 10^6 cells.

To determine the levels of dATP and dCTP, and the effect of clofarabine on these deoxynucleotides, Ficoll-enriched blasts were extracted with 60% methanol and the dNTPs were quantitated using a modified DNA polymerase assay as described previously.21,22

**Results**

**Patient demographics**

Characteristics of the 18 adults (median age, 51 y; range, 27-67 y) who were enrolled and treated with clofarabine-CY are delineated in Table 1. All patients had refractory leukemia with multiple poor-risk features and had received multiple prior therapies. Median peripheral blood blast count for the entire group was 14 × 10^9/L (range, 0.8 × 10^9/L to 65 × 10^9/L), with the median for AML patients being 12 × 10^9/L and ALL patients being 29 × 10^9/L. Of 12 AML patients, 8 (67%) were primary refractory AML, 2 were refractory at second relapse, and 2 had first CR for less than 6 months. Similarly, 3 (50%) of 6 ALL patients had disease recurrence while on maintenance therapy and 2 relapsed after SCT (1 allogeneic, 1 autologous). The majority (14/18, 78%) had adverse cytogenetics,23-25 including 10 (83%) of 12 AML23,25 and 4 (67%) of 6 ALL patients (3 complex, 1 Ph^- ALL).24 Six patients were treated at dose level 1 (clofarabine 20 mg/m²/daily dose, CY 400 mg/m²/daily dose) and 12 were treated at dose level 0 (clofarabine 10 mg/m²/daily dose, CY 400 mg/m²/daily dose). Three of the 12 AML and 3 of the 6 ALL patients received dose level 1, and the remainder (9 AML and 3 ALL) received dose level 0. The decrease in clofarabine dose was prompted by the occurrence of prolonged marrow aplasia (≥day 60) in 2 patients (1 AML, 1 ALL) and death in 2 patients from hepatic toxicity, prolonged marrow aplasia (1 ALL), and early onset multiorgan failure (1 AML; Table 2). These issues are discussed further in “Results, Toxicities.”

**Toxicities**

The initial dose of CY 200 mg/m² on day 0 induced a pronounced (≥50%) drop in peripheral blasts in only 1 of the 18 patients on study, whereas 5 patients (3 ALL, 2 AML) had at least 20% increases. Following day 1 clofarabine-CY administration, blast counts fell by at least 50% in 12 (67%) patients (5/6 [83%] dose level 1, and 7/12 [58%] dose level 0). By day 4, following completion of all 3 doses of clofarabine-CY, all patients had at least a 50% decrease in peripheral blasts and 15 (83%) had at least a 90% decrease. There was no statistical difference noted between the dose levels.

Table 2 details toxicity profiles for both dose levels. The initial dose of CY 200 mg/m² on day 0 induced a pronounced (≥50%) drop in peripheral blasts in only 1 of the 18 patients on study, whereas 5 patients (3 ALL, 2 AML) had at least 20% increases. Following day 1 clofarabine-CY administration, blast counts fell by at least 50% in 12 (67%) patients (5/6 [83%] dose level 1, and 7/12 [58%] dose level 0). By day 4, following completion of all 3 doses of clofarabine-CY, all patients had at least a 50% decrease in peripheral blasts and 15 (83%) had at least a 90% decrease. There was no statistical difference noted between the dose levels.

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there was no clear relationship between the occurrence of tumor lysis and the level of circulating blasts or the rate of fall in blasts in response to either CY or clofarabine-CY. One AML patient at dose level 1 experienced transient grade 3 respiratory distress. However, no patient required pressor support, renal dialysis, or mechanical ventilation during the initial tumor lysis period. Unlike other intensive antileukemia regimens with nucleoside analogs, anthracyclines, antimetabolites, and/or topoisomerase II–directed agents, oral and/or gastrointestinal mucositis were both uncommon and mild. Hepatic dysfunction as evidenced by grade 3 or lower transaminits or hyperbilirubinemia or both occurred at both clofarabine doses but with somewhat greater frequency at the higher clofarabine dose (4/6 dose level 1 vs 2/12 dose level 0). Hepatic function returned to baseline values within 7 days in all but 1 patient at dose level 1 who died from multiorgan failure.

The median time to marrow recovery for patients treated on dose level 1 was 45 days (range, 24 to >100 d). Hematologic DLT consisted of profound marrow aplasia lasting longer than 60 days in 2 of the 6 dose level 1 patients; 1 of who died with profound marrow aplasia and concomitant transaminits on day 100, and the other who remained aplastic until day 75, at which time she recovered with initial trilineage hematopoiesis followed by recurrent leukemia (days 55, 58).

Clinical outcome

Table 3 summarizes the clinical outcome after 1 cycle of clofarabine-CY. Responses occurred at both dose levels. The first response assessment occurred on day 14 of therapy, when marrow was assessed for presence or absence of residual leukemia by routine marrow aspirate, biopsy, and flow-cytometry. CTC was documented in 4 (67%) of 6 patients at dose level 1 and in 8 (67%) of 12 at dose level 0. An additional patient (T-cell ALL) treated at dose level 0 had markedly reduced marrow blasts from 90% to 10%. CR was achieved in 2 (33%) of 6 dose level 1 patients, both with ALL (pre-B-ALL with complex cytogenetics including 6q–, relapsed after allogeneic SCT in CR2; Ph+ pre-B-ALL refractory to induction therapy plus imatinib). Of the 12 patients treated at dose level 0, 3 (25%) achieved CR including 1 primary refractory AML, 1 refractory AML with 3 prior induction regimens, and 1 pre-B-ALL relapsed on maintenance therapy with hyperleukocytosis and CNS involvement. An additional patient with T-ALL who relapsed on maintenance therapy achieved PR, for an overall response rate of 33% at dose level 0. Patients treated at both dose levels who achieved CR or PR were able to receive 1 to 3 additional cycles of clofarabine-CY (2 AML, 1 T-ALL), allogeneic SCT (2 AML), or donor lymphocyte infusion (DLI; 1 ALL), whereas patients who achieved PR (1 AML) or who did not have sustained response to a single cycle of clofarabine-CY (4 AML) were able to receive additional investigational salvage regimens.

DNA damage

Previous in vitro studies have demonstrated the inability of CLL cells to fully repair CY-induced DNA damage when those cells are pretreated with clofarabine.13 To determine if clofarabine could have similar effects in vivo, we compared the presence and magnitude of damaged DNA by flow-cytometric measurement of
γH2AX in peripheral blood blasts obtained longitudinally from 13 patients prior to day 0 at end of CY infusion (EOI), 2 hours after CY EOI, and on day 1 of clofarabine-CY 2 hours after CY EOI. As depicted in Figure 1 and Table 4, γH2AX increased immediately following day 0 (200 mg/m²) CY in 5 (38%) patients, with at least 3-fold increases seen in 2 (15%), but did not increase further over the next 2 hours. Administration of clofarabine prior to the day 1 dose of CY in the same patients, however, led to marked increases in γH2AX relative to day 0 pretreatment values in 12 (92%) of 13 patients, with 7 (54%) of 13 having striking (≥3-fold) increases that persisted for at least 2 hours after the end of the day 1 CY infusion (Figure 2). Moreover, cells obtained on day 1 from 11 (85%) of 13 patients evinced 2.4- to 38-fold increases in γH2AX relative to the comparable time point on day 0 (ie, 2 hours after CY EOI; Figure 2). For the 9 patients who achieved day 14 CTC, γH2AX in blasts obtained after the day 1 CY infusion increased a median 14.5-fold (range, 0.3-37 fold) relative to day 0 levels, with 6 (67%) having at least a 3-fold increase. In contrast, only 1 of the 3 patients who had residual leukemia on day 14 had at least a 3-fold increase (1.5, 1.7, 38) relative to day 0 γH2AX levels.

Bone marrow blasts were obtained longitudinally from 4 patients on day 0 EOI CY and day 3 EOI clofarabine-CY. As depicted in Figure 3, there was no consistent increase in the amount of γH2AX detected in marrow blasts following the initial dose of CY on day 0. Following the day 3 doses of clofarabine-CY, however, the amount of detectable DNA damage was magnified in all 4 patients, with 2 patients having dramatic (> 40-fold) increases in γH2AX.

## Apoptosis

The overall fraction of peripheral blood blasts undergoing apoptosis, as measured by the amount of sub-2N DNA in the blast population, increased 1.2- to 1.6-fold at all time points relative to day 0 pretreatment levels (Table 4). Nonetheless, 8 (62%) of 13 patients evinced at least a 1.5-fold increase in the sub-2N fraction following clofarabine-CY, whereas such increase was detected in 4 (32%) of 13 following CY alone. Median increase in day 1 post–clofarabine-CY sub-2N DNA was 2.2-fold (range, 0.2- to 62-fold) for the 9 patients achieving day 14 CTC and 1.4-fold for 3 with residual leukemia. Interestingly, all 3 patients who achieved CTC and CR had at least a 1.5-fold increase in sub-2N DNA on day 1 (1.6, 1.8, 2.0). In contrast, of the 4 patients who achieved CTC but were ultimately no response (NR), only 1 had a greater than 1.5-fold increase and 2 had major decreases (0.2-fold) in sub-2N DNA. The patient with the greatest increase in sub-2N DNA (62-fold increase over day 0) died of irreversible bone marrow failure.

### Intracellular clofarabine metabolism

Clofarabine-TP accumulation was measured at the end of the clofarabine infusion in 4 patients, with intracellular clofarabine-TP levels being 6, 24, 28, and 50 pmol/10⁷ cells. Ribonucleotides (ATP, CTP, GTP, TTP) were not affected by clofarabine treatment. For dNTPs, due to limited cell numbers, we measured dATP and dCTP only. The endogenous ICTP concentration was between 5 and 56 pmol/10⁷ cells (n = 6). There was little effect on these values after clofarabine infusion.

### Table 4. In vivo effects of clofarabine on cyclophosphamide-induced DNA damage and apoptosis in peripheral blood blasts

<table>
<thead>
<tr>
<th></th>
<th>Day 0, CY alone</th>
<th>Day 1, clofarabine → CY, 2 h after end of infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fold increase in γH2AX relative to pretreatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>1.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Range</td>
<td>0.4-6.2</td>
<td>0.6-6.8</td>
</tr>
<tr>
<td>No. of 1.5-fold or greater increase (%)</td>
<td>5/13 (38)</td>
<td>3/13 (23)</td>
</tr>
<tr>
<td>No. of 3-fold or greater increase (%)</td>
<td>2/13 (15)</td>
<td>1/13 (8)</td>
</tr>
</tbody>
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| **Fold increase in cells with sub-2N DNA content** |                |                                              |
| Median                 | 1.3            | 1.2                                          |
| Range                  | 0.5-52         | 0.4-90                                       |
| No. of 1.5-fold or greater increase (%) | 3/13 (23)     | 4/13 (32)                                     |

Where applicable, data are no./total no. of patients in that category.
and heavily pretreated group of adults with acute leukemias, a single cycle of clofarabine followed by CY yielded clinical responses in 3 of (25%) 12 AML patients and 4 (67%) of 6 ALL patients. The net response rates in this small group of patients do not appear to relate to clofarabine dose. On the other hand, the achievement of day 14 marrow CTC and CR may relate to the amount of DNA damage achieved with clofarabine-CY, a hypothesis that needs further study in more patients in order to be validated. This notion is consistent with the observation that toxicity was to some extent dose related, particularly with regard to prolonged marrow aplasia. Nonetheless, there may be some apparent uncoupling of net DNA damage and apoptosis in some patients, particularly those who achieved CTC on day 14 but ultimately recovered with leukemia (NR). These findings are not surprising and suggest that additional pathways for DNA repair and net cellular survival may be operative in these poor-prognosis patients and may circumvent the enhancement of CY-induced DNA damage by clofarabine.

Clofarabine followed by CY was associated with increases in DNA damage and apoptosis in both AML and ALL blasts from both the peripheral blood and the marrow. The damage was measured by phosphorylation of the histone variant H2AX, an early event that occurs in response to DNA double-strand breaks. Data from longitudinal studies conducted in 13 patients suggest an increase in phosphorylation and accumulation of γH2AX after addition of clofarabine to CY, consistent with the notion that the addition of clofarabine augmented net CY-induced DNA damage. Since this work was performed in a limited number of patients to achieve its phase 1 objectives, a more extensive study will be needed to confirm these observations.

The inhibition of DNA synthesis may be due to inhibition of RR and incorporation of clofarabine-TP in the DNA repair patch. In this analysis of limited patient sample numbers, we observed no decrease in dATP during therapy, which may suggest a limited role of RR inhibition. However, in our study, we did not measure the effect of clofarabine on other dNTP pools. In this regard, in vivo studies accompanying a clinical trial of clofarabine given prior to the classic nucleoside analog ara-C detected depletion of intracellular cytosine pools by clofarabine and a resultant increase in ara-CTP incorporation into DNA. The in vivo data recapitulated the in vitro finding that clofarabine potentiated the intracellular metabolism of ara-C in leukemia cell line model systems. In our study, there was a narrow sample range and limited laboratory end points, making it difficult to define the total effect of clofarabine on CY-induced DNA damage and any resultant repair response. It will be of interest to examine these parameters in other diseases and in clofarabine combinations with other DNA-damaging agents.

Nucleoside analogs such as fludarabine, pentostatin, and cladribine have been combined effectively with CY in lymphoid malignancies such as CLL. Clinical data in patients who failed single-agent therapies with either nucleoside analogs or alkylating agents demonstrate that these patients can respond to the combination of such agents with an overall response rate of roughly 40%, suggesting that the combination may lower or eliminate resistance to each agent when used individually.

The combination of clofarabine with the classic alkylating agent CY, which damages DNA directly through adduct formation, is a new combination strategy based on preclinical studies in leukemia cells. At least in theory, this mechanism-based combination requires the presence of clofarabine-TP during the DNA repair phase of CY-induced DNA damage. Thus, an
In sum, clofarabine followed by CY induces significant leukemia cell kill in patients with refractory AML and ALL on both the molecular and clinical levels. The activity of this combination warrants further exploration in patients with relapsed and refractory acute leukemias. The toxicities of this regimen are noteworthy and suggest that modifications in total doses of one or both drugs may afford equal efficacy while improving tolerability.

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Authorship

Contribution: J.E.K. designed and performed research, analyzed data, and wrote the paper; R.M.R. performed laboratory assays for DNA damage and apoptosis, prepared specimens for pharmacokinetic studies, and helped to analyze data and write the paper; K.B. conducted pharmacokinetic analysis including clofarabine triphosphate accumulation and intracellular dATP levels; J.B. coordinated clinical drug administration and sample acquisition for laboratory correlates; J.G. performed research, collected data, and assisted with data analysis; S.D.G., B.D.S., M.A.M., H.C., and M.J.L. participated in protocol design, performed research, and helped to analyze data; and V.G. participated in protocol design, directed pharmacokinetic laboratory end points, and helped to write the paper. All authors reviewed and approved the manuscript prior to submission.

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References


A phase 1 clinical-laboratory study of clofarabine followed by cyclophosphamide for adults with refractory acute leukemias

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