Cytarabine-resistant Leukemia Cells Are Moderately Sensitive to Clofarabine *In Vitro*

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Abstract. *Background/Aim: Clofarabine is transported into leukemic cells via the equilibrative nucleoside transporters (hENT) 1 and 2 and the concentrative nucleoside transporter (hCNT) 3, then phosphorylated by deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) to an active triphosphate metabolite. Cytarabine uses hENT1 and dCK for its activation. We hypothesized that cytarabine-resistant leukemia cells retain sensitivity to clofarabine. Materials and Methods: Human myeloid leukemia HL-60 cells and cytarabine-resistant variant HL/ara-C20 cells were used in the present study. Results: Despite 20-fold cytarabine resistance, the HL/ara-C20 cells exhibited only a 6-fold resistance to clofarabine compared to HL-60 cells. The intracellular concentration of the triphosphate metabolite of cytarabine was reduced to 1/10, and that of clofarabine was halved in the HL/ara-C20 cells. hENT1 and dCK were reduced, but hCNT3 and dGK were not altered in the HL/ara-C20 cells, which might contribute to their retained capability to produce intracellular triphosphate metabolite of clofarabine. Conclusion: Clofarabine was cytotoxic to leukemia cells that were resistant to cytarabine.*

Key agents for the treatment of leukemia have included purine and pyrimidine nucleoside analogs for over 30 years. Cytarabine (1-β-D-arabinofuranosylcytosine) is the mainstay for treating acute myeloid leukemia (AML) (1-5). Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine) is a key agent for the treatment of chronic lymphocytic leukemia (6), and cladribine (2-chloro-2'-deoxyadenosine) is used for the treatment of hairy-cell leukemia (7). Clofarabine [2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)adenine] (Clolar™ ; Genzyme, Cambridge, MA, USA) is a relatively new purine nucleoside analog (5, 8-10). The rationale behind its design was to combine the structural features of fludarabine and cladribine (6, 7). Clofarabine is cytotoxic against non-proliferating human lymphocytes and rapidly-proliferating cells (5). In 2004, the U.S. Food and Drug Administration approved clofarabine for the treatment of pediatric patients with acute lymphoblastic leukemia (ALL) (8-11). The anticancer activities of clofarabine toward various types of tumors are being investigated in other age groups and for other types of leukemia, including AML (12-14).

Upon administration, clofarabine is transported into leukemia cells through two types of membrane nucleoside transporters: human equilibrative nucleoside transporters (hENT) 1 and 2, and human concentrative nucleoside transporter (hCNT) 3 (15). Inside the cells, clofarabine is phosphorylated to its mono-phosphate derivative by a cytoplasmic enzyme, deoxycytidine kinase (dCK) and by a mitochondrial enzyme, deoxyguanosine kinase (dGK). Further intracellular phosphorylation results in the production of the active metabolite, clofarabine triphosphate, which is then incorporated into DNA, terminating DNA elongation and eventually inducing apoptosis (16-19).

Nucleoside analogs exert cytotoxicity similarly through the inhibition of DNA synthesis, but the activation pathways differ between cytarabine and clofarabine (1, 16-19). Clofarabine is incorporated into leukemia cells *via* hENT1, -2, and hCNT3, while cytarabine is transported into cells through hENT1. Clofarabine is phosphorylated by both dCK and dGK, and cytarabine is phosphorylated by dCK. Om this regard, clofarabine might still be efficacious against leukemia that becomes refractory to treatment using cytarabine-based regimens.

We hypothesized that leukemia cells that acquired resistance against cytarabine would be sensitive to clofarabine because of differences in the activation pathways. The present study evaluated the *in vitro* cytotoxicity of clofarabine against the human myeloid leukemia HL-60 cell line and a HL-60 variant cell line that we previously established to be resistant to cytarabine (20). The mechanisms of intracellular activation were investigated.

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Key Words: Clofarabine, clofarabine triphosphate, drug resistance, cytarabine, acute myeloid leukemia.

Materials and Methods

Drugs and chemicals. Clofarabine was kindly provided by Genzyme (Cambridge, MA, USA). Cytarabine was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. The human acute leukemia HL-60 cells and cytarabine-resistant HL-60 variant, HL/ara-C20, established in our laboratory (20), were cultured in RPMI1640 media supplemented with 10% fetal bovine serum at 37˚C in humidified atmosphere with 5% CO_2.

Proliferation assay. To evaluate the growth-inhibitory effects, the sodium 3'-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN, USA) with slight modifications (20).

Quantification of drug-induced apoptosis. To measure cytotoxicity, drug-induced apoptosis was measured. After being treated with different concentrations of clofarabine or cytarabine, the cells were stained with propidium iodide and subjected to cell-cycle analysis using flow cytometry. Apoptotic cell death was quantitated as an increase in the sub-G_1 fraction (21).

Western blot analysis. To determine the protein levels of hENT1, hCNT3, dCK, and dGK, western blotting was performed. A mouse antibody against dCK that was provided by Dr. Hori at Mie University School of Medicine (22), a rabbit polyclonal antibody against dGK (Abcam, Cambridge, UK), a rabbit polyclonal antibody against hENT1 (Santa Cruz Biotechnology, Dallas, TX, USA), a rabbit polyclonal antibody against hCNT3 (Santa Cruz Biotechnology), and a rabbit polyclonal antibody against actin (Sigma, St Louis, MO, USA) were used as against primary antibodies.

Determination of intracellular analog triphosphates. To quantify the intracellular concentrations of clofarabine triphosphate and cytarabine triphosphate, a high-performance liquid chromatography (HPLC) method previously established in our laboratory was used (23, 24). Cells (1×10^6 /ml, 20 ml) were incubated with 10 μM clofarabine or 10 μM cytarabine for 6 h at 37˚C. The intracellular analog triphosphate concentrations were expressed as pmol/10^7 cells.

Statistical analyses. All graphs were generated with GraphPad, Prism software (version 5.0; GraphPad Software, Inc. San Diego, CA, USA).

Results

Growth inhibition and the induction of apoptosis by clofarabine. The growth-inhibitory effects of clofarabine were compared between HL-60 cells and the cytarabine-resistant HL/ara-C20 variant cells. The HL/ara-C20 cells were 20-fold more resistant to cytarabine than were the HL-60 cells (Figure 1A, Table I). The 50% growth-inhibitory concentration (IC_{50}) values indicate that the relative resistance to clofarabine is 6-fold despite the 20-fold resistance to cytarabine in the HL/ara-C20 cells (Figure 1B, Table I).

Table I. *Drug sensitivities in HL-60 and HL/ara-C 20 cells.*

Drugs	IC_{50} (nM)		
	HL-60	HL/ara-C20	RR
Ara-C	335	5300	(20)
Cl-F-ara-A	50	320	(6.4)

The HL-60 cells and HL/ara-C20 cells were incubated with various concentrations of cytarabine (1-β-D-arabinofuranosylcytosine, ara-C) or clofarabine (2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl) adenine, Cl-F-ara-A) for 72 h. The 50%-growth-inhibitory concentration (IC_{50}) was then determined by using the sodium 3'-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])- bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay. The number in the parenthesis is the relative resistance (RR), which was obtained by dividing the IC_{50} value of the HL/ara-C20 cells by that of the HL-60 cells.

To investigate the drug-induced cytotoxicity, the cells were evaluated for induction of apoptosis (Figure 2). When cells were incubated with clofarabine or cytarabine at a concentration that was compatible with the IC_{50} for each agent in each cell line, clofarabine induced greater apoptosis than did cytarabine in both cell lines (Figure 2). These results suggest that cytarabine-resistant cells were moderately sensitive to clofarabine and that clofarabine was more cytotoxic than cytarabine against both cell lines.

Determination of intracellular clofarabine triphosphate metabolites. The surrogate for the cytotoxicity of nucleoside analogs is an intracellular triphosphate form. When the cells were treated with 10 μM cytarabine for 6 h, the intracellular concentration of the cytarabine triphosphate metabolite in the HL/ara-C20 cells (77±9 pmol/10^7 cells) was 1/10 of the concentration in the HL-60 cells (646±5 pmol/10^7 cells) ($p<0.0001$, Student's *t*-test) (Figure 3A). When the cells were treated with 10 μM clofarabine for 6 h, the amount of intracellular clofarabine triphosphate metabolite in the HL/ara-C20 cells (28±7 pmol/10^7 cells) was half that in HL-60 cells (55±19 pmol/10^7 cells) ($p=0.06$, Student's *t*-test) (Figure 3B). The reduction in clofarabine triphosphate metabolite was smaller than the reduction in cytarabine triphosphate metabolite in HL/ara-C20 cells, which would contribute to the moderate cytotoxicity of clofarabine retained in HL/ara-C20 cells.

Membrane transporters and kinases. The membrane nucleoside transporters and kinases are most closely associated with the intracellular analog triphosphate production (23-26). Western blot analysis demonstrated a reduced expression of hENT1 and dCK in HL/ara-C20 cells compared to their expression in the HL-60 cells (Figure 4). The attenuated production of cytarabine triphosphate might be attributable to

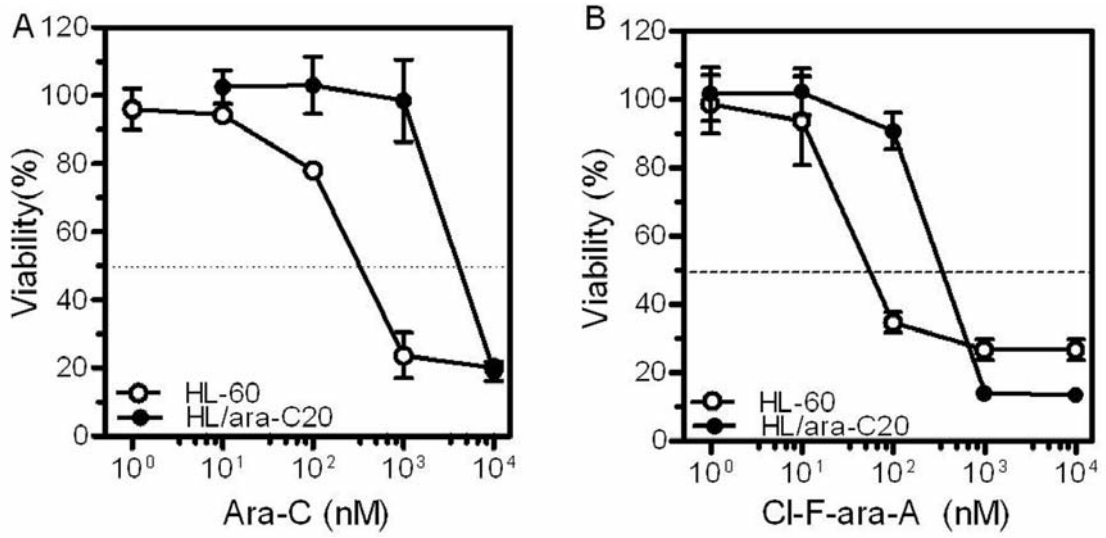

Figure 1. *HL-60 cells or HL/ara-C20 cells were incubated with different concentrations of cytarabine (ara-C) (A) or clofarabine (Cl-F-ara-A) (B) for 72 h. The incubation was followed by evaluation of proliferation using the sodium 3'-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate assay. The values are means±SD of at least three independent experiments.*

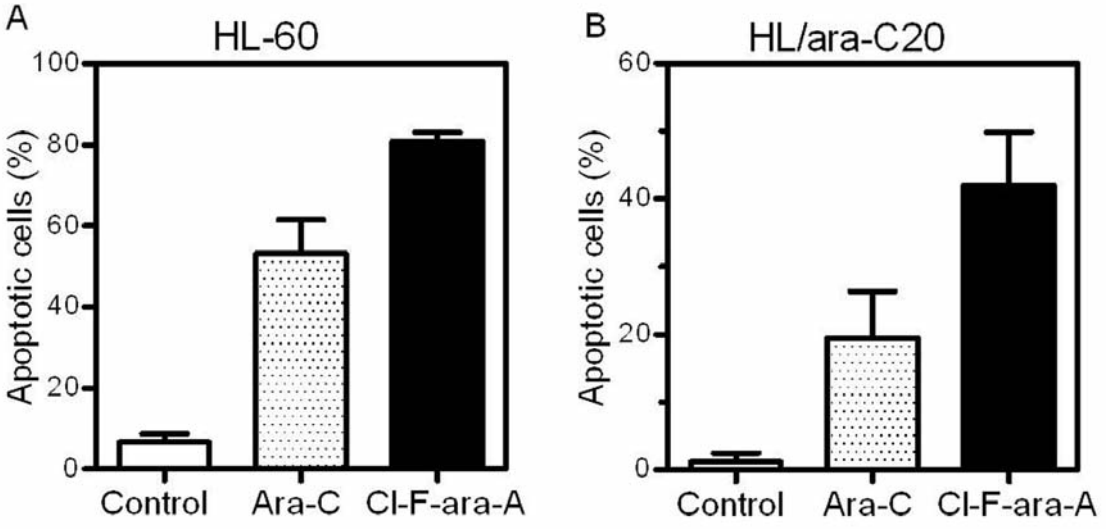

Figure 2. *The induction of apoptosis. A: HL-60 cells were incubated with 300 nM cytarabine (ara-C) or 50 nM clofarabine (Cl-F-ara-A) for 48 h. B: HL/ara-C20 cells were incubated with 5,000 nM cytarabine or 300 nM clofarabine for 48 h. Both cell lines were evaluated for the induction of apoptosis using flow cytometry. The values are means±SD of at least three independent experiments.*

these reductions in HL/ara-C20 cells. hCNT3 and dGK were not altered in the HL/ara-C20 cells (Figure 4), and these are not associated with the intracellular activation of cytarabine. These results suggest that the HL/ara-C20 cells retained their capability for generating the triphosphate metabolite of clofarabine, likely because clofarabine was incorporated into the cells through hCNT3 and subsequently phosphorylated to the clofarabine nucleotide by dGK.

Discussion

Cytarabine is a pyrimidine nucleoside analog and a key agent used for the treatment of AML (4, 27). Long-term survivors account for only 40-50% of the young adult patients with AML who are treated with cytarabine-based chemotherapy. The 5-year survival rates in elderly patients are only a third of those seen in younger patients (28, 29). Acquired

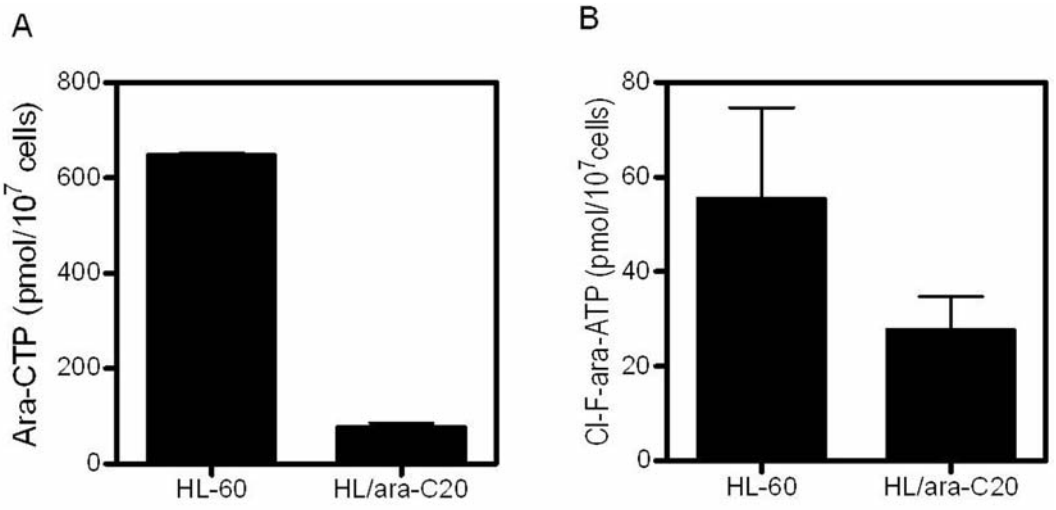

Figure 3. *Triphosphate metabolite production. HL-60 cells and HL/ara-C20 cells were incubated with 10 μM cytarabine (ara-C) or 10 μM clofarabine (Cl-F-ara-A) for 6 h. The incubation was followed by evaluation of the intracellular triphosphate metabolite of cytarabine (ara-CTP) (A) or clofarabine (Cl-F-ara-ATP) (B) using HPLC. The values are means±SD of at least three independent experiments.*

resistance to anticancer agents is a major obstacle for successful cancer treatment, and overcoming resistance to cytarabine would offer new strategies for the treatment of AML. In the present study, the cytarabine-resistant variant HL/ara-C20 cells held moderate sensitivity to a similar nucleoside analog, clofarabine (Table I, Figure 1). Clofarabine induced a greater amount of apoptosis in HL-60 and in the HL/ara-C20 cells than did cytarabine (Figure 2). HL/ara-C20 cells had the capability for generating intracellular clofarabine triphosphate (Figure 3), which might be attributable to intact hCNT3 and dGK (Figure 4). These results suggest that clofarabine may exert cytotoxicity against AML that is refractory to cytarabine.

The production of an intracellular triphosphate form is crucial for the cytotoxicity of nucleoside analogs. The factors that contribute to the efficient production of analog triphosphates include membrane nucleoside transporters and kinases. Clofarabine enters cells by facilitated and active nucleoside transport mechanisms and by passive diffusion across lipid membranes (15, 16). Transport *via* hENTs is sodium-independent, bi-directional facilitative diffusion, while hCNTs are sodium-dependent, ATP-dependent active transporters. Inside the cells, clofarabine is phosphorylated to its monophosphate form primarily by dCK, a constitutively expressed key cytosolic enzyme involved in the salvage pathway of DNA synthesis, and by the mitochondrial enzyme dGK. (16). The cellular activation mechanism of cytarabine is similar to, but slightly different from, the mechanism of clofarabine (1). Cytarabine is imported into leukemia cells *via* hENT1 and does not use hCNT3. In cells, it is phosphorylated to cytarabine monophosphate by dCK,

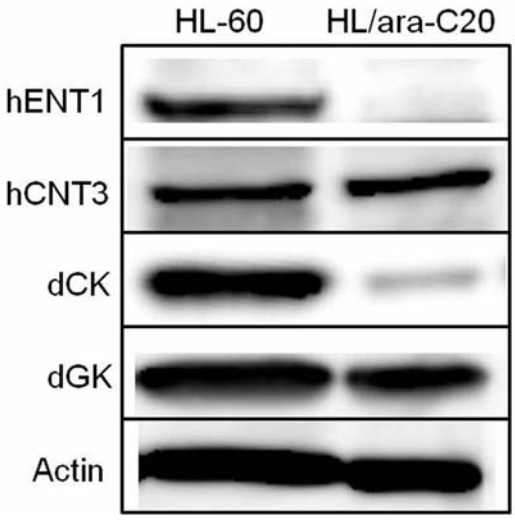

Figure 4. *Protein expression of the membrane nucleoside transporters and kinases. The expression levels of human equilibrative nucleoside transporter (hENT) 1, human concentrative nucleoside transporter (hCNT) 3, deoxycytidine kinase (dCK), and deoxyguanosine kinase (dGK), were evaluated using western blot analysis.*

but not by dGK. The difference in the activation pathway might avoid complete cross-resistance between clofarabine and cytarabine.

In our previous study, two clofarabine-resistant HL-60 variants were established, and their mechanism of resistance was elucidated (22). The two variants were 20-fold and 80-fold more clofarabine-resistant than HL-60, respectively. The

mRNA levels of *hENT1* and *hCNT3* were reduced, and the protein levels of dCK and dGK were attenuated in both resistant variants. The subsequent production of clofarabine triphosphate was decreased in these variants. The 20-fold more resistant variant exhibited 568-fold greater resistance to cytarabine, and the 80-fold resistant variant showed much higher cytarabine resistance, the IC_{50} of which was in excess of 20 μM that was used as a maximum concentration for performing the XTT assay. These results suggest that once leukemia cells acquire resistance to clofarabine, they are no longer sensitive to cytarabine. Leukemia cells that become resistant to cytarabine might still retain sensitivity to clofarabine.

A phase I, multi-center study was conducted to assess the maximum-tolerated dose, safety, pharmacokinetics, and efficacy of clofarabine in Japanese adults with AML (30). In the present study, clofarabine was given to the patients at doses ranging from 20 mg/m^2 to 50 mg/m^2 as 1-h intravenous infusions for five consecutive days. The maximum clofarabine concentrations in the plasma ranged between 1 μM and 4 μM. The IC_{50} value of clofarabine for the HL/ara-C20 cells (320 nM) (Table I) was achievable clinically, so clofarabine would be efficacious for treating patients with AML who experience relapse after cytarabine-based chemotherapy and were suspected to have acquired resistance to cytarabine.

Burnett *et al*. reported the efficacy of clofarabine in untreated older patients with AML. Clofarabine was administered as monotherapy by a 1-h infusion of 30 mg/m^2 daily on days 1 through 5. A total of 106 patients were treated, and 48% had a complete response (31). Agura *et al*. reported on therapeutic efficacy of cytarabine and clofarabine in combination, for the treatment of patients with relapsed/refractory AML and for elderly patients with untreated AML and heart disease (32). The patients received five days of clofarabine (40 mg/m^2) and cytarabine (1,000 mg/m^2). Among 30 patients, the overall response rate (complete remission plus partial remission) was 53%, including a complete remission in 14 patients (47%). The present study may support the basic rationale for the use of clofarabine against refractory/relapsed AML. Clofarabine may be suitable as second-line treatment for AML that relapses after cytarabine-based chemotherapy.

Disclosure Statement

The Authors have nothing to disclose concerning any of the drugs or agents used in the present study.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23501307, 2010).

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Received December 29, 2013
Revised February 2, 2014
Accepted February 3, 2014