Fludarabine-Mediated Circumvention of Cytarabine Resistance Is Associated with Fludarabine Triphosphate Accumulation in Cytarabine-Resistant Leukemic Cells

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Fludarabine-Mediated Circumvention of Cytarabine Resistance Is Associated with Fludarabine Triphosphate Accumulation in Cytarabine-Resistant Leukemic Cells

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Abstract

The combination of cytarabine (ara-C) with fludarabine is a common approach to treating resistant acute myeloid leukemia. Success depends on a fludarabine triphosphate (F-ara-ATP)-mediated increase in the active intracellular metabolite of ara-C, ara-C 5′-triphosphate (ara-CTP). Therapy-resistant leukemia may exhibit ara-C resistance, the mechanisms of which might induce cross-resistance to fludarabine with reduced F-ara-ATP formation. The present study evaluated the effect of combining ara-C and fludarabine on ara-C–resistant leukemic cells in vitro. Two variant cell lines (R1 and R2) were 8-fold and 10-fold more ara-C resistant, respectively, than the parental HL-60 cells. Reduced deoxycytidine kinase activity was demonstrated in R1 and R2 cells, and R2 cells also showed an increase in cytosolic 5′-nucleotidase II activity. Compared with HL-60 cells, R1 and R2 cells produced smaller amounts of ara-CTP. Both variants accumulated less F-ara-ATP than HL-60 cells and showed cross-resistance to fludarabine nucleoside (F-ara-A). R2 cells, however, accumulated much smaller amounts of F-ara-ATP and were more F-ara-A resistant than R1 cells. In HL-60 and R1 cells, F-ara-A pretreatment followed by ara-C incubation produced F-ara-ATP concentrations sufficient for augmenting ara-CTP production, thereby enhancing ara-C cytotoxicity. No potentiation was observed in R2 cells. Nucleotidase might preferentially degrade F-ara-A monophosphate over ara-C monophosphate, leading to reduced F-ara-ATP production and thereby compromising the F-ara-A–mediated potentiation of ara-C cytotoxicity in R2 cells. Thus, F-ara-A–mediated enhancement of ara-C cytotoxicity depended on F-ara-ATP accumulation in ara-C–resistant leukemic cells but ultimately was associated with the mechanism of ara-C resistance.

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1. Introduction

Cytarabine (1-β-D-arabinofuranosylcytosine) (ara-C), a pyrimidine nucleoside analogue, is among the most effective anticancer agents in both induction and salvage chemotherapies for acute myeloid leukemia [1,2]. The first step in the mechanism is the transportation of ara-C into leukemic cells by membrane transporters, including the human equilibrative nucleoside transporter 1 (hENT1) [3]. Ara-C is then phosphorylated to ara-C 5′-monophosphate by deoxycytidine kinase, the rate-limiting enzyme, and subsequently to ara-C 5′-triphosphate (ara-CTP) [4-7]. Ara-CTP is partly incorporated as a monophosphate into DNA strands during the S phase of the cell cycle [6,7]. Drug incorporation into DNA inhibits the extending primer from further incorporation of deoxyribonucleotides, including deoxycytidine triphosphate (dCTP), thereby inhibiting DNA synthesis [8-10]. Because the amount of drug incorporated into DNA is the product of the ara-CTP concentration and time, ara-CTP is an index of ara-C cytotoxicity [8-10]. The clinical application of this index was established by documenting a correlation between the intracellular pharmacokinetics of ara-CTP and the response to ara-C therapy [11-14]. Thus,

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in vitro and clinical studies have suggested the utility of therapeutic strategies that augment ara-CTP production in leukemic blasts.

One strategy that increases the intracellular ara-CTP concentration is pretreatment with the purine nucleoside analogue fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate) [15-17]. Fludarabine nucleoside (F-ara-A) is taken up by leukemic cells and is phosphorylated into the active form, fludarabine 5′-triphosphate (F-ara-ATP), through the pathway common to ara-C [18]. F-ara-ATP stimulates deoxycytidine kinase in 2 ways, thereby enhancing ara-CTP production. The first is a direct effect on deoxycytidine kinase. The second effect is indirect and is mediated through the inhibition of ribonucleotide reductase, an enzyme responsible for the de novo synthesis of deoxyribonucleotides [19]. The inhibition of ribonucleotide reductase produces a decline in the intracellular dCTP pool, thereby decreasing dCTP-mediated feedback inhibition of deoxycytidine kinase [15-17]. In both cases, the intracellular F-ara-ATP concentration is critical to ara-CTP enhancement. On the basis of these in vitro findings, a combination-chemotherapy regimen consisting of fludarabine, ara-C, and granulocyte colony-stimulating factor, designated as FLAG, has been developed for clinical use [20,21]. This regimen and similar regimens further combined with an anthracycline have been safely used to achieve a nearly 50% rate of complete remission in patients with chemoresistant acute myeloid leukemia [22-25].

Not all patients with refractory or relapsed acute myeloid leukemia respond to FLAG therapy, however, because some leukemic cells acquire drug resistance, including resistance to the key agent, ara-C. Resistance to ara-C in leukemic cells primarily involves a reduction in the intracellular concentration of ara-CTP [8-14]. The mechanisms responsible for the decrease in the ara-CTP concentration include reduced drug uptake and decreased deoxycytidine kinase activity. Increased cytidine deaminase and cytosolic 5′-nucleotidase II activities also contribute to ara-C resistance [26-28]. Because F-ara-A and ara-C share the same pathway for cellular uptake and subsequent phosphorylation, most mechanisms responsible for ara-C resistance also decrease F-ara-ATP production [29-31]. If so, F-ara-A–mediated enhancement of ara-CTP production might be compromised in patients with chemoresistant leukemia. Overcoming this limitation requires a better understanding of the biochemical interaction between ara-C and F-ara-A in the context of ara-C resistance.

The present investigation evaluated the in vitro effect of F-ara-A and ara-C in combination on 2 ara-C–resistant leukemic cell lines. Specifically, we studied the modulation effects of F-ara-A on dCTP and ara-CTP concentrations and on subsequent cytotoxicity.

2. Materials and Methods

2.1. Chemicals and Reagents

Ara-C, F-ara-A, and nitrobenzylthioinosine (NBMPR) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [5-^{3}H]ara-C (30 Ci/mmol) was purchased from Daiichi Pure Chemicals (Tokyo, Japan). Inosine 5′-monophosphate (IMP) labeled with [8-^{14}C]IMP (45-60 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Tetrahydrouridine was purchased from Calbiochem (La Jolla, CA, USA). *L*-(+)-rhamnose, $NaIO_4$, and methylamine were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytic grade.

2.2. Development of Ara-C–Resistant Leukemic Cell Lines

Human leukemia HL-60 cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum at 37°C in humidified air containing 5% carbon dioxide. To develop ara-C–resistant HL-60 variants, we cultured parental HL-60 cells independently in 2 separate flasks in media containing ara-C. The initial ara-C concentration was half the 50%–growth-inhibitory concentration (IC_{50}) of HL-60 cells. The cultures were observed daily and allowed to grow. Drug concentrations were gradually increased on subsequent passages, and a single ara-C–resistant cell line was cloned from each flask by the limiting-dilution method. The 2 independent ara-C–resistant HL-60 variants were named R1 and R2.

2.3. Proliferation Assay

To evaluate the proliferation of each cell line, we seeded medium with HL-60, R1, and R2 cells at a density of 1×10^5/mL, allowed them to grow, and counted cell numbers every 24 hours up to 72 hours by means of the trypan blue dye-exclusion assay. The growth-inhibitory effect of nucleoside analogues was evaluated with the sodium 3′-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) assay according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA) [32]. In brief, we incubated 1 mL of cells (5×10^4/mL) for 24 hours in a 24-well plate and then added a 10-μL aliquot of different concentrations of ara-C or F-ara-A. The cells were incubated for the next 72 hours, and a 100-μL aliquot was transferred to a 96-well microplate. The cells then were mixed with 50 μL XTT and incubated for another 4 hours at 37°C. Spectrophotometry analysis was performed at an absorbance of 480 nM with a fluorescence microplate reader, (SpectraMax 250; Molecular Devices Japan, Ashiya, Japan). The IC_{50} value was extrapolated from a growth-inhibition curve produced for each drug.

2.4. Determination of Intracellular Production of Analogue Triphosphates

To evaluate the intracellular production of ara-CTP and F-ara-ATP, we incubated HL-60, R1, and R2 cells (1×10^6/mL, 10 mL) with different concentrations of ara-C or F-ara-A for discrete intervals. To a cell pellet (1×10^7 cells) obtained by centrifugation (14,900*g*, 20 seconds), we added 30 μL of 15% perchloric acid. The sample was vortexed, cooled in an ice bath for 15 minutes, and centrifuged again (15,000*g*, 30 seconds at 4°C). The supernatant was neutralized with potassium hydroxide and obtained as an acid-soluble fraction, the nucleotide pool [11]. The acid-soluble fraction was applied to an ion-exchange column (TSK-Gel

DEAE-2 SW, 250-mm length × 4.6-mm inside diameter; Tosoh, Tokyo, Japan) for high-performance liquid chromatography (HPLC). Elution was carried out at ambient temperature with 0.05 M Na_2HPO_4 (pH 6.9)/20% acetonitrile at a constant flow rate of 0.7 mL/min. Ara-CTP and F-ara-ATP peaks were identified separately under the same HPLC conditions [33] and quantitated by the peak areas at absorbances of 269 nm and 261 nm, respectively. The values were expressed as pmol/10^7 cells. The packed cell volume (1×10^7 HL-60 cells) was measured in triplicate to determine the micromolar concentration for comparing the present data with historical controls.

2.5. Nucleoside-Transport Capacity

To evaluate the capacity of the membrane nucleoside transporter, we quantified nucleoside analogue uptake in all cell lines by the method of Wiley et al, with slight modifications [3]. In brief, cells (1×10^7/mL, 1 mL) were incubated for 5 minutes at 20°C, followed by pulse treatment with 1 μM tritiated ara-C (specific activity, 15 μCi/μmol)) for 60 seconds. The samples were quickly overlaid on oil (300 μL) in a microcentrifuge tube and centrifuged (14,900*g*, 20 seconds at 4°C) to terminate the reaction. The supernatant and oil interface were completely removed. The cell pellet was mixed with 1 mL 0.5 N sodium hydroxide for 12 hours at 45°C to solubilize the pellet. Sample radioactivities were measured with a scintillation counter on the following day. Nonfacilitated drug uptake was determined in the presence of 3 μM NBMPR, which interferes with the membrane nucleoside transporter. The capacity of the transporter was determined as the difference in drug uptake in the absence and presence of NBMPR.

2.6. Measurement of Deoxycytidine Kinase and Cytosolic 5′-Nucleotidase II

Deoxycytidine kinase activity was assayed as previously described, with slight modifications [34]. In brief, we obtained crude enzyme by sonicating cells suspended in 100 μL 50 mM Tris-HCl (pH 8.0) containing 50% glycerol and then clarifying the enzyme preparation by centrifugation (100,000*g*, 60 minutes at 4°C). The enzyme (20 μL) was incubated for 30 minutes at 37°C in a reaction buffer (total volume, 60 μL) containing 20 μM [5-^{3}H]ara-C (specific activity, 500 μCi/μmol), 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM adenosine 5′-triphosphate (ATP), 12.5 mM dithiothreitol, and 1 mM tetrahydrouridine. The reaction was terminated by placing the sample in an ice bath. A 10-μL aliquot of the sample was spotted on a cellulose thin-layer chromatography sheet (Polygram Cel 300 UV_{254}, 20 cm × 20 cm × 0.1 mm; Macherey-Nagel, Düren, Germany) and developed for 3 hours with solvent (water–2-propanol–acetic acid, 1:2:2 vol/vol). The plate was cut into 1-cm strips, which were then put in scintillation vials filled with 10 mL Clear-sol I (Nacalai Tesque). Radioactivity was counted on the following day. Enzyme activity was expressed in picomoles per hour per milligram protein.

The activity of cytosolic 5′-nucleotidase II was measured as previously described, with slight modifications [30]. The enzyme (20 μL) obtained as described above was mixed with reaction buffer (total volume, 60 μL) containing 50 mM imidazole (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.5 g/L bovine serum albumin, 0.2 mM α,β-methylene adenosine diphosphate (which inhibited the activity of membrane-bound 5′-ectonucleotidase), 5 mM β-glycerophosphate, 100 μM EGTA, and 200 μM IMP and [8-^{14}C]IMP (specific activity, 500 μCi/μmol). ATP (3 mM) was added to activate cytosolic 5′-nucleotidase II. The reaction was allowed to continue for 30 minutes at 37°C and terminated by placing the sample in an ice bath. A 10-μL aliquot of the sample was applied to a thin-layer chromatographic sheet as described above. Enzyme activity was expressed in picomoles per minute per milligram protein.

2.7. Determination of dCTP Concentration

To determine the intracellular dCTP concentration, we extracted the acid-soluble fraction as described above and subjected the extract to the periodate-oxidation procedures originally described by Garrett and Santi [35]. In brief, the acid-soluble fraction was mixed with 20 μL 0.5 M $NaIO_4$ and 50 μL 4 M methylamine (pH adjusted to 7.5 with phosphoric acid), and incubated for 30 minutes at 37°C to degrade ribonucleotides. The sample then was added to 10 μL 1 M rhamnose and put in an ice bath for 15 minutes to remove the $NaIO_4$. The mixture was applied to an HPLC column as described above, and the dCTP concentration (pmol/10^7 cells) was quantitated by its peak area at an absorbance of 254 nm [33].

2.8. Biochemical Interaction between Ara-C and F-ara-A

To determine the effect of F-ara-A, we preincubated HL-60, R1, and R2 cells (1×10^6/mL, 10 mL) with 10 μM F-ara-A for 3 hours, washed the cells, resuspended them in fresh media, and further incubated the cells with 10 μM ara-C for 3 hours. The cells were washed to terminate the reaction and resuspended again in fresh media. Intracellular ara-CTP and dCTP concentrations were determined immediately by HPLC, and cell proliferation was evaluated with the XTT assay after 72 hours.

2.9. Statistical Analyses

Nonparametric Mann-Whitney *U* tests were performed with StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA), and all graphs were generated with GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA). A *P* level of ≤.05 was defined as statistically significant.

3. Results

3.1. Development of 2 Ara-C–Resistant HL-60 Variants and Measurement of Intracellular Ara-CTP Concentrations

The IC_{50} values for ara-C in HL-60 cells and cells of the 2 ara-C–resistant variants (R1 and R2) were compared. The

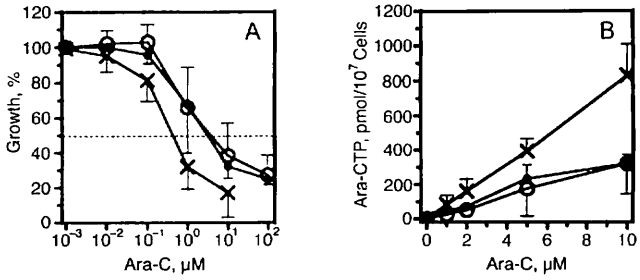

Figure 1. A, Growth-inhibition effect of cytarabine (ara-C) on HL-60 cells and 2 ara-C–resistant variants (R1 and R2). Cells were incubated for 72 hours with or without different concentrations of ara-C. Proliferation was determined with the XTT assay. Values are presented as the mean ± SD of at least 3 independent experiments for HL-60 (X), R1 (●), and R2 (O) cells. B, Intracellular concentrations of ara-C 5′-triphosphate (ara-CTP). HL-60, R1, and R2 cells were incubated for 6 hours with ara-C (0, 1, 2, 5, or 10 μM), followed by nucleotide pool extraction and subsequent measurement of ara-CTP. Values are presented as the mean ± SD of at least 3 independent experiments for HL-60 (X), R1 (●), and R2 (O) cells.

XTT assay demonstrated that R1 and R2 cells were comparably more ara-C resistant than HL-60 cells (Figure 1A, Table 1). The magnitude of the ara-C resistance in these cells was relatively low and might be clinically relevant. The growth rates of the cell lines were similar (Table 1), suggesting that resistance to this S phase–specific drug was not attributable to the speed of the cell cycle. When the cells were incubated with ara-C, intracellular ara-CTP production was increased in a concentration-dependent manner in all cell lines (Figure 1B). During a 6-hour incubation with 10 μM ara-C, HL-60 cells produced more ara-CTP (830 pmol/10^7 cells) than R1 cells (322 pmol/10^7 cells; $P = .025$, Mann-Whitney U test) and R2 cells (321 pmol/10^7 cells; $P = .05$, Mann-Whitney U test) (Figure 1B). R1 and R2 cells were similar with respect to Ara-CTP production. Intracellular ara-CTP concentration appeared to correlate with the magnitude of ara-C resistance (Figure 1).

Table 1.
Drug Sensitivities of HL-60 and 2 Cytarabine (Ara-C)-Resistant Variants (R1 and R2) to Ara-C*

		Ara-C	
	Doubling Time, h	IC_{50}, μM	Relative Resistance
HL-60 cells	14.2	0.4	
R1 cells	13.5	3.2	8
R2 cells	13.1	4.0	10

*HL-60, R1, and R2 cells were incubated with various ara-C concentrations for 72 hours and evaluated for proliferation with the XTT assay. The 50%-inhibitory concentration (IC_{50}) is the mean of at least 3 independent experiments. The relative resistance value was obtained by dividing the IC_{50} values for R1 and R2 cells by the IC_{50} value for HL-60 cells. Doubling time was determined with the trypan blue dye-exclusion assay.

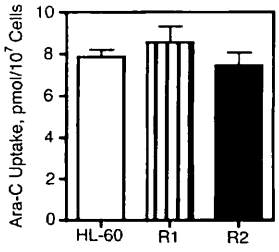

Figure 2. The capacity of the membrane nucleoside transporter in HL-60, R1, and R2 cells. After pulse treatment with 1 μM tritiated cytarabine (ara-C) for 60 seconds, drug uptake was determined by scintillation counting. Values are presented as the mean ± SD of at least 3 independent experiments.

3.2. Membrane Nucleoside-Transport Capacity

To elucidate the mechanisms of ara-C resistance, we determined the capacity of the membrane nucleoside transporter. When cells were pulsed with ara-C, the drug was rapidly incorporated in all 3 cell lines (Figure 2). Analogue uptakes in HL-60, R1, and R2 cells were similar (Figure 2), suggesting that the membrane nucleoside-transport capacity was not associated with the development of ara-C resistance.

3.3. Deoxycytidine Kinase and Cytosolic 5′-Nucleotidase II

To further elucidate the mechanisms of ara-C resistance, we assayed both deoxycytidine kinase and cytosolic 5′-nucleotidase II activities. Deoxycytidine kinase activity was lower in R1 and R2 cells than in HL-60 cells ($P = .0495$, R1 versus HL-60; $P = .0495$, R2 versus HL-60; Mann-Whitney U tests) (Figure 3A). This lower activity might be responsible for reduced ara-CTP production. The similar kinase activities in R1 and R2 cells ($P = .52$, Mann-Whitney U test) might reflect the comparably reduced ara-CTP production in these 2 cell lines. Cytosolic 5′-nucleotidase II activities in HL-60 and R1 cells were the same ($P = .51$, Mann-Whitney U test), whereas the enzyme activity of R2 cells was higher than that of HL-60 cells ($P = .021$, Mann-Whitney U test) and R1 cells ($P = .049$, Mann-Whitney U test; Figure 3B). Thus, we demonstrated reduced deoxycytidine kinase activity in R1 and R2 cells and increased cytosolic 5′-nucleotidase II activity in R2 cells. Table 2 summarizes the mechanisms of ara-C resistance in the 2 resistant variants.

3.4. Sensitivity to F-ara-A and Measurement of Intracellular F-ara-ATP Concentrations

We compared F-ara-A IC_{50} values for HL-60, R1, and R2 cells. Both ara-C–resistant variants showed cross-resistance to F-ara-A; however, R2 cells were more F-ara-A resistant than R1 cells despite similar degrees of ara-C resist-

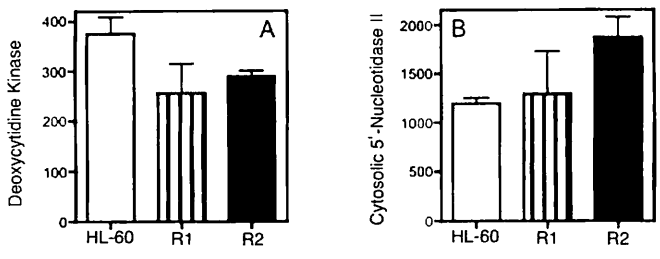

Figure 3. Deoxycytidine kinase (A) and cytosolic 5′-nucleotidase II (B) activities. Deoxycytidine kinase activity was determined in cell extracts from HL-60 and the resistant variants (R1, R2) with cytarabine (ara-C) as a substrate and is expressed in picomoles per hour per milligram protein. Cytosolic 5′-nucleotidase II activity was determined in the HL-60 and resistant variants (R1, R2) cell lines with inosine 5′-monophosphate as a substrate in the presence of adenosine 5′-triphosphate and is expressed in picomoles per minute per milligram protein.

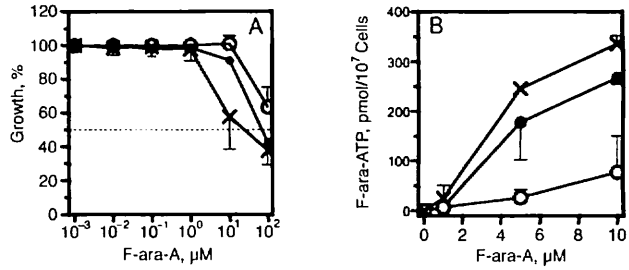

Figure 4. A, Growth inhibition of fludarabine nucleoside (F-ara-A) on HL-60 cells and 2 cytarabine (ara-C)-resistant variants (R1 and R2 cells). Cells were incubated with or without F-ara-A at different concentrations for 72 hours. Proliferation was determined with the XTT assay. Values are presented as the mean ± SD of at least 3 independent experiments for HL-60 (X), R1 (●), and R2 (O) cells. B, Intracellular fludarabine 5′-triphosphate (F-ara-ATP) concentrations. HL-60, R1, and R2 cells were incubated for 3 hours with F-ara-A (0, 1, 5, or 10 μM), followed by nucleotide pool extraction and subsequent measurement of F-ara-ATP. Values are presented as the mean ± SD of at least 3 independent experiments for HL-60 (X), R1 (●), and R2 (O) cells.

ance (Figure 4A, Table 3). When cells were incubated with F-ara-A, intracellular F-ara-ATP production was increased in a concentration-dependent manner in all cell lines (Figure 4B). During a 3-hour incubation with 10 μM F-ara-A, HL-60 cells produced more F-ara-ATP (335 pmol/10^7 cells) than R1 cells (267 pmol/10^7 cells; $P = .049$, Mann-Whitney U test) and R2 cells (77 pmol/10^7 cells; $P = .049$, Mann-Whitney U test) (Figure 4B). F-ara-ATP production by R1 cells was greater than by R2 cells ($P = .049$, Mann-Whitney U test), suggesting that the ability to accumulate F-ara-ATP varied despite comparable reductions in ara-CTP concentration in these 2 cell lines. Cytosolic 5′-nucleotidase II is purine specific and has recently been reported to show a very low activity with ara-C monophosphate [36,37]. Enhanced nucleotidase activity might dephosphorylate F-ara-A monophosphate more efficiently than ara-C monophosphate, thereby leading to a lower production of F-ara-ATP by R2 cells. Thus, R2 cells with an increased cytosolic 5′-nucleotidase II activity accumulated a smaller amount of F-ara-ATP, which led to the greater magnitude of F-ara-A resistance compared with R1 cells (Figure 4).

3.5. Alteration of Intracellular dCTP and Ara-CTP Concentrations and Enhancement of Ara-C Cytotoxicity by F-ara-A

To determine the effect of the combination of F-ara-A and ara-C on ara-C–resistant leukemic cells, we incubated HL-60, R1, and R2 cells with 10 μM F-ara-A for 3 hours, followed by washing in fresh media and subsequent incubation with 10 μM ara-C for 3 hours. F-ara-A pretreatment reduced the dCTP concentration by 40% in HL-60 cells (Figure 5A) and augmented ara-CTP production 1.8-fold (Figure 5B). F-ara-A pretreatment was more cytotoxic than ara-C alone, with cell viability reduced from 68% to 48% (a 29% reduction) ($P = .05$, Mann-Whitney U test; Figure 5C). A 3-hour incubation with 10 μM F-ara-A alone minimally inhibited cell growth (data not shown). The results suggested that the ara-CTP augmentation was directly associated with the enhancement of the growth-inhibitory effects of ara-C in ara-C–sensitive HL-60 cells.

In R1 cells, preincubation with 10 μM F-ara-A similarly reduced the dCTP level by 33% (Figure 5A) and increased ara-CTP production 1.9-fold (Figure 5B). The subsequent cytotoxicity also was enhanced, with the viability reduced from 90% to 66% (a 27% reduction) ($P = .05$, Mann-Whitney U test; Figure 5C). The equipotent enhancement of cytotoxicity in HL-60 and R1 cells might be attributed to comparably augmented ara-CTP production. In R2 cells, preincubation with 10 μM F-ara-A did not alter dCTP and ara-CTP concentrations (Figures 5A and 5B). Cytotoxicity was not enhanced by F-ara-A in R2 cells, a result that might be due to the unstimulated ara-CTP production.

The packed cell volume of 1×10^7 HL-60 cells was 21.2 ± 0.9 μL (mean ± SD of 3 independent determinations). Therefore, the F-ara-ATP concentrations produced by 10 μM F-ara-A in HL-60 and R1 cells (335 and 267 pmol/10^7 cells, respectively) were equivalent to concentrations of 12 to 16 μM. These F-ara-ATP concentration values are consistent with concentrations (≥10 μM) previously shown to maximally augment ara-CTP production in acute myeloid leukemia blasts [38]. However, the F-ara-ATP concentration produced in R2 cells was 3.6 μM (77 pmol/10^7 cells), which did not surpass this threshold.

Table 2.
Mechanisms of Cytarabine (Ara-C) Resistance

	Deoxycytidine Kinase	Cytosolic 5′-Nucleotidase II	Uptake*
R1	↓	→	→
R2	↓	↑	→

*Cellular uptake of ara-C.

Table 3.
Drug Sensitivities of HL-60 and 2 Cytarabine (Ara-C)-Resistant Variants (R1 and R2) to Fludarabine Nucleoside (F-ara-A)*

	F-ara-A	
	IC_{50}, μM	Relative Resistance
HL-60 cells	22.0	
R1 cells	70.0	3.5
R2 cells	>100.0	—

*HL-60, R1, and R2 cells were incubated with various F-ara-A concentrations for 72 hours and evaluated for proliferation with the XTT assay. The 50%-inhibitory concentration (IC_{50}) was determined as the mean of at least 3 independent experiments. The relative resistance value was obtained by dividing the IC_{50} values for R1 and R2 cells by the IC_{50} value for HL-60 cells. The F-ara-A IC_{50} value was not determined for R2 cells because of the limited solubility of F-ara-A.

Thus, F-ara-A–mediated potentiation of ara-C cytotoxicity was compromised in ara-C–resistant R2 cells, with reduced F-ara-ATP accumulation due to increased cytosolic 5′-nucleotidase II activity.

4. Discussion

Overcoming resistance to ara-C would offer new strategies for the treatment of acute myeloid leukemia. The present study examined the interaction between ara-C and F-ara-A, the biochemical rationale for FLAG chemotherapy, on 2 ara-C–resistant leukemic cell lines, with the focus on changes in intracellular dCTP and ara-CTP concentrations and enhancement of growth inhibition. The effectiveness of this combination was viewed from the perspective of ara-C resistance mechanisms.

Ara-C–resistant HL-60 variants R1 and R2 were cross-resistant to F-ara-A (Figure 4A, Table 3). Mechanisms of F-ara-A resistance include decreased deoxycytidine kinase activity and increased ribonucleotide reductase and cytosolic 5′-nucleotidase II activities [26,29-31]. Changes in deoxycytidine kinase and/or cytosolic 5′-nucleotidase II activities associated with ara-C–resistant variants (Figure 3) would then reasonably be expected to induce cross-resistance to F-ara-A. Nevertheless, R1 and R2 cells exhibited different F-ara-A sensitivities despite similar degrees of ara-C resistance (Figures 1A and 4A, Tables 1 and 3). This result was closely associated with the varying ability to accumulate F-ara-ATP (Figure 4B) despite comparable reductions in ara-CTP production (Figure 1B). The major difference between R1 and R2 cells was the increased activity of cytosolic 5′-nucleotidase II in R2 cells (Figure 3B). Cytosolic 5′-nucleotidase II activity is reportedly associated with ara-C resistance in vitro [26] and reduced clinical efficacy [39,40]. The present study confirms that ara-C–resistant leukemic cells can show increased activity of this enzyme. However, cytosolic 5′-nucleotidase II is purine specific and has a minimal effect on ara-C monophosphate dephosphorylation [36,37]. Schirmer et al demonstrated that cladribine-resistant HL-60 cells that possessed increased cytosolic 5′-nucleotidase II activity and intact deoxycytidine kinase activity lacked cross-resistance to gemcitabine and ara-C [41]. This absence was consistent with the purine specificity of the enzyme. Therefore, increased cytosolic 5′-nucleotidase II activity in R2 cells might preferentially degrade F-ara-A monophosphate over ara-C monophosphate and thereby reduce F-ara-ATP accumulation, explaining why R2 cells were more F-ara-A resistant than R1 cells.

Previous studies demonstrated that F-ara-A pretreatment enhanced the rate of ara-CTP accumulation in a human leukemic cell line in vitro [15]. Clinical studies have confirmed the effectiveness of this combination [16,17,38]. The fludarabine-induced increase in the rate of ara-CTP accumulation was dependent on the cellular concentration of F-ara-ATP, and 10 μM F-ara-ATP was the threshold for maximizing ara-CTP augmentation (2-fold increase) in leukemic blasts [38]. In the present study, HL-60 and R1 cells pretreated with 10 μM F-ara-A achieved intracellular F-ara-ATP concentrations (12-16 μM) that exceeded this threshold (Figure 4B). These F-ara-ATP concentrations were also greater than the concentration (8.5 μM) needed for the 50% inhibition of cytidine diphosphate reduction by ribonucleotide reductase in HeLa cells [42]. Accordingly, dCTP was reduced, ara-CTP was augmented (1.8-fold to 1.9-fold increase), and successful potentia-

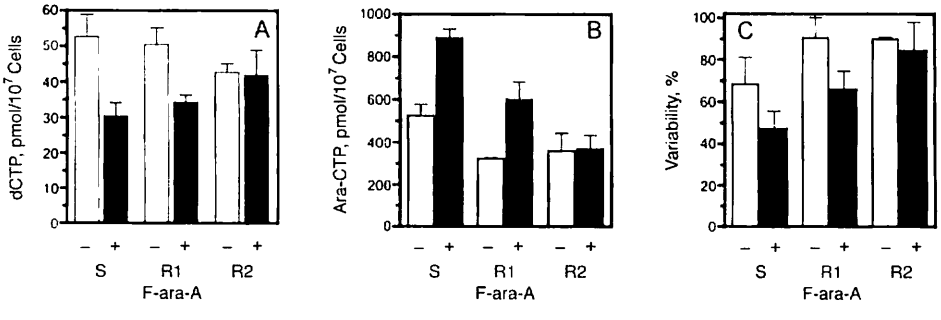

Figure 5. The biochemical interaction between cytarabine (ara-C) and fludarabine nucleoside (F-ara-A). The experimental setting is detailed in "Materials and Methods." The effects of preincubation with (+) and without (–) 10 μM F-ara-A on intracellular deoxycytidine triphosphate (dCTP) (A) and cytarabine 5′-triphosphate (ara-CTP) (B) concentrations and on subsequent cytotoxicity (C) were evaluated. Values are presented as the mean ± SD of at least 3 independent experiments. S indicates HL-60 cells.

tion of ara-C cytotoxicity was obtained (Figure 5). The degrees of enhanced cytotoxicity in HL-60 and R1 cells were almost identical (Figure 5C), suggesting that F-ara-A could enhance ara-C cytotoxicity despite the ara-C resistance of the cells. In contrast to R1 cells, no potentiation was observed in R2 cells (Figure 5); this result might be due to the reduced accumulation of F-ara-ATP to below the threshold. The decreased F-ara-ATP level might be due to an elevation in cytosolic 5′-nucleotidase II activity in R2 cells; however, this explanation suggests that intracellular F-ara-ATP concentration varies with the mechanism of ara-C resistance. In this sense, F-ara-ATP could be a surrogate marker for predicting the F-ara-A–mediated potentiation of ara-C cytotoxicity. Thus, success of the biochemical interaction between ara-C and F-ara-A in ara-C–resistant leukemic cells depended on the accumulation of intracellular F-ara-ATP, but it ultimately was associated with the mechanisms of ara-C resistance.

FLAG therapy is effectively used to treat patients with refractory or relapsed acute myeloid leukemia. However, some leukemic blasts might not respond to fludarabine-mediated potentiation because ara-C resistance might collaterally reduce F-ara-ATP production. Our findings thus suggest ways to identify patients who are candidates for combination therapy with ara-C and fludarabine. In vitro testing to determine ara-CTP augmentation by incubating patients' leukemic cells with ara-C in the presence and absence of F-ara-A might be conducted prior to initiating chemotherapy.

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