Comparison of cytotoxicity of 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine (clofarabine) with cladribine in mononuclear cells from patients with acute myeloid and chronic lymphocytic leukemia

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Background and Objectives. Clofarabine (CAFdA), one of the newer nucleoside drugs is undergoing a phase II clinical trial for the treatment of pediatric refractory/relapsed acute myeloid and lymphocytic leukemia. Although CAFdA is structurally similar to the clinically established analogs fludarabine and cladribine (CdA), its metabolism and mechanism of actions are significantly different. The present study investigates the in vitro cytotoxicity of CAFdA and CdA in mononuclear cells isolated from 52 patients with chronic lymphocytic (CLL) and acute myeloid leukemia (AML).

Design and Methods. We incubated the leukemic cells with drugs for 48 hours and cytotoxicity was then evaluated by the MTT dye assay. We also determined the levels of deoxycytidine and deoxyguanosine kinase with radio-chemical substrate-based assays and used a high performance liquid chromatographic method to measure cellular nucleotides in leukemia cells after 2 hours' incubation.

Results. Using equimolar concentrations of CAFdA and CdA, the in vitro cytotoxicity for the population was significantly higher with CAFdA than with CdA (median EC50 for CAFdA 0·12 µM and for CdA 0·15 µM, p<0·001). From the individual estimates the difference in cytotoxicity between CAFdA and CdA was more pronounced in cells from CLL patients (median EC50 for CAFdA 0·08 µM and for CdA 0·16 µM p<0·001) than in those from AML patients. We also found that CAFdA was phosphorylated more efficiently than CdA. No correlations were detected in this study between the levels of CdA and CAFdA nucleotides, enzymes levels and the in vitro responses.

Interpretation and Conclusions. The greater in vitro cytotoxicity and cell metabolism of CAFdA compared to CdA confirm the high activity of CAFdA and encourage clinical trials with CAFdA in leukemic patients.

Key words: cladribine, clofarabine, deoxycytidine kinase, deoxyguanosine kinase, leukemia, in vitro cytotoxicity.

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The purine analogs used in the treatment of low-grade hematologic malignancies include cladribine (2-chlorodeoxyadenosine, CdA, Leustatin®) and fludarabine (Fluoroadenine-β-D-arabinoside, Fludara®) (Figure 1). Their unique characteristics in comparison with traditional antimetabolites is that they are equally cytotoxic to both dividing and resting cells in vitro. CdA has been documented to have therapeutic activity in hairy cell leukemia, B-CLL, low grade B-cell lymphomas, cutaneous T-cell lymphoma and acute myeloid leukemia as well as in the treatment of multiple sclerosis and rheumatoid arthritis.1

2-chloro-2'-arabino-fluoro-2'-deoxyadenosine (clofarabine, CAFdA, Clofarex™, Figure 1) is a 2'-arabino-fluoro derivative of CdA and fludarabine with promising cytotoxicity in human cell lines and leukemia-bearing mice with severe combined immunodeficiency.2 CAFdA is more acid stable than are CdA and fludarabine3 and, using isolated perfused rat liver, we have previously demonstrated that CAFdA is resistant to deglycosylation by hepatic enzymes.4 Encouraging results from phases I clinical trials of this drug have recently been reported in patients with solid and hematologic disorders5-7 and it is currently undergoing a phase II clinical trial for the treatment of pediatric refractory/relapsed acute myeloid and lymphocytic leukemia. Like CdA and fludarabine, CAFdA is phosphorylated by the key salvage enzyme deoxycytidine kinase (dCK) and by mitochondrial deoxyguanosine kinase (dGK).8 After further cellular phosphorylation nucleoside triphosphates compete with endogenous DNA or RNA precursors for incorporation into nucleic acids, leading to blocked proliferation or apoptosis. The importance of dGK in vivo is not known. High dCK levels and low 5’-nucleotidase levels have previously been shown to correlate with therapeutic effect of CdA in CLL9,10 However, the formation of CAFdA-nucleotides in vitro or in vivo has not been shown to correlate with the clinical effects of CdA.11-13 The aim of the present study was to assess the in vitro cytotoxicity of leukemic cells to CAFdA and CdA. We also delineated the relationship between the activity of phosphorylating enzymes, the cellular level of phosphorylated metabolites, and the in vitro cytotoxicity of CdA and CAFdA.

Design and Methods

Patients and treatment

Fifty-two patients with leukemia, 31 with B-chronic lymphocytic leukemia (CLL) and 21 with acute myeloid...
leukemia (AML, classification according FAB criteria; 1 M0, 5 M1, 5 M2, 4 M4, 1 M5, 3 M5A, 2 M5B) (Table 1) were studied. Twenty-five of the patients had not received any treatment before the start of the study, three patients had received CdA treatment, none had received fludarabine and twenty-four had received treatment with other cytostatic drugs (generally low-dose chlorambucil for CLL and intensive combination chemotherapy for AML). The local Ethics Committee at the Karolinska Hospital approved the study (Dnr 97-326).

Chemicals and reagents
Dr. Zygmunt Kazimierczuk synthesized the CdA and CdAMP at the Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland. CAFdA was a gift from Dr. Howard Cottam, University of California, San Diego, CA, USA. Fludarabine was a gift from Sheering AG. Cytarabine (Cytosar®) was purchased as pharmaceutical formulations from the Karolinska Hospital pharmacy. Lymphoprep was purchased from Nycomed, Oslo, Norway. RPMI 1640 medium, heat-inactivated fetal calf serum, L-glutamine and penicillin-streptomycin were all from Gibco, Life Technologies, Paisley, UK. 3-4,5 dimethylthiazol-2.5 diphenyl tetrazolium bromide (MTT) was from Sigma Chemical Company, St. Louis, MO, USA. Other compounds were of analytical grade and purchased from Sigma.

Design and Methods

Processing of patients’ samples
One venous sample (10 mL) was collected from each patient from an indwelling catheter into a heparinized Venoject® tube, before drug intake. Mononuclear cells were isolated by standard Lymphoprep (1.077 g/mL) density centrifugation (500xg, 25 min). After separation on Lymphoprep and washing with phosphate-buffered saline, the cell pellet was dissolved in 2 mL of ice-cold distilled water and after 30 seconds 1 mL of 2.7% NaCl was added and then the volume was adjusted to 50 mL with phosphate-buffered saline. This procedure was adjusted to lyse erythrocytes and reticulocytes in order to avoid interference. The number of cells in the samples and their median cell volume were determined by a Coulter Multisizer (Coulter Electronics, Luton, UK).

Incubation conditions and determination of nucleotides
The isolated mononuclear cells (25×10⁶) were incubated with 200 nM CdA (which corresponds to the plasma concentration achieved during a 2h infusion of 0.12 mg/kg) and 200 nM CAFdA for 2 h at 37°C. After incubation, nucleotides were extracted from leukemia cells by the perchloric acid method as previously described. Intracellular concentrations of CdA and CAFdA nucleotides were determined using high performance liquid chromatography on an Ultrasphere ODS column (250×4.6 mm, 5 µm; Beckman Instruments, Fullerton, CA, USA) and quantified at 265 nm. The intracellular concentrations of nucleotides were calculated and expressed as the quantity of nucleotides contained in the extract from a given number of cells of a determined mean volume.

Activity measurements of dCK and dGK
Enzymes activities were measured in extracts of freshly isolated leukemia cells using CdA as a substrate according to a previously published procedure. Cells were suspended at 1-2×10⁶ cells/100 µL in an extraction buffer containing 50 mM Tris-HCl pH 7.6, 2 mM diethiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% Nonidet P40, frozen and thawed three times and centrifuged at 11,800xg in an Eppendorf centrifuge.
for 5 min at 4°C. The supernatant was collected and used as a source of protein for the enzyme assays. The protein content in cell extracts was determined according to protocol (BC protein assay, Bio-Rad Laboratories, CA, USA). CdA (50 µM, 10 times higher than the K<sub>v</sub> value) with specific radioactivities of 500-1000 cpm/pmol was used as substrate. The activity of dGK was determined by using \([8-3H]-CdA\) in the presence of deoxycytidine (500 µM) to block the dCK enzyme. The assays were initiated by addition of 2-3 µg proteins to a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM ATP, 4 mM dithiothreitol, 10 mM sodium fluoride and substrates. Aliquots were withdrawn and spotted on WATMAN DE81 papers. Filters were then washed as described earlier, eluted by 50 µL of 0.4 M perchloric acid and counted in 3 mL Ecocint scintillation fluid in a liquid scintillation counter (RackBeta, LKB Wallac, Turku, Finland). The conditions to maintain a linear, reaction rate were determined in preliminary experiments. The enzyme activities were expressed as pmol/mg cellular protein/min (pmol/mg/min).

**Cytotoxicity test**

The drug sensitivity was measured by determination of the concentration inducing a 50% effect/inhibition of cell growth (EC<sub>50</sub>) in a MTT assay, as previously reported. Briefly, 252,000 cells for CLL patients and 126,000 cells for AML patients, were incubated in triplicate for 48 h with increasing concentrations of compounds in a 96-well plate in a final volume of 100 µL. Cells were incubated for 4 h in the presence of MTT and after dissolving the crystals in 10% lauryl sulphate sodium salt/10 mM HCl overnight, the optical density was read at 540/690 nm.

The drug concentrations for CdA and CAFdA were 0.02-20 µM while those for cytarabine and fludarabine were 1.0-204 µM. To analyze the concentration-effect relation thoroughly, an expanded concentration range of CdA and CAFdA (0.0001-100 µM) and of fludarabine and cytarabine (0.01-100 µM) were tested in samples from ten patients.

An assay was judged adequate for analysis when the control optical density was >0.050 (as also used by Pieters et al., 1990). Only coefficients of variation (CV) less than 25% for a triplicate and 20% for the control wells were accepted. The MTT assay was technically successful in 101/106 plates (95%) and 511/583 drug triplets (88%). The inter-assay coefficient of variation was less than 6.5%.

**Evaluation of cytotoxicity test**

In the initial, exploratory analysis, a non-linear relationship between drug concentration and optical density was suggested and a sigmoid inhibitory effect model with a baseline effect parameter (Equation 1) was used to describe the relationship.

**Equation 1**

\[
E = E_0 \times \left(1 - \frac{E_{\text{max}} \times C^\gamma}{C^\gamma + EC_{50}^\gamma}\right)
\]

Abbreviations: \(E=\text{effect}, E_{\text{max}}=\text{maximal effect}, E_0=\text{baseline value}, C=\text{drug concentration}\)

The drug concentration and effect, measured by optical density, was evaluated by non-linear mixed effects modeling. For each drug, data from all individuals were analyzed simultaneously together with data from baseline experiments without the drug.

The parameters estimated in the non-linear mixed effects models were: \(E_{\text{max}}, EC_{50}\) and the slope \((\gamma)\). For all three parameters the typical value in the studied group was estimated and was the magnitude of interindividual variability. In addition, individual empirical Bayes estimates of \(E_{\text{max}}\) and \(EC_{50}\) were estimated. The estimates of \(E_{\text{max}}\) and \(EC_{50}\) for CdA and CAFdA were used to identify relationships with covariates, dCK and dGK expression, intracellular concentrations of CdAMP, CdATP, CAFdAMP and CAFdATP, leukocyte particle concentration, cell size and the patient’s age, sex and diagnosis. These individual parameters were regressed on the potential covariates using a generalized additive model.

**Statistical analysis**

Statistics analysis was performed using the STATISTICA for Windows release 5.1 from StatSoft, Inc., Tulsa, OK, USA. As the data were not normally distributed and the sample size was small, non-parametric methods were used. In addition to the generalized additive modeling, the correlations between enzyme activity, drug \(EC_{50}\) and nucleotide phosphorylation were evaluated using Spearman’s rank order correlation. The individual predictions of \(EC_{50}\) from the population were used in the correlation analysis. In analysis of the relationship between the activity of dCK and dGK a \(p\) value less than 0.05 was regarded as statistically significant. Due to multiple analyses, a \(p\) value less than 0.001 was regarded as statistically significant for the rest of the correlation analyses. The difference between CdA and CAFdA nucleotides was analyzed by the Wilcoxon matched pairs test and difference between CLL and AML patients as well as the difference between untreated and refractory/relapsed patients by the Mann-Whitney U test. A \(p\) value of less than 0.05 was considered to be statistically significant. All data are presented as median values with ranges in parentheses. The graphs were drawn using GraphPad Prism version 3.0a for Apple Macintosh® (GraphPad Software, San Diego CA, USA). The data analysis and the simulations of the
final model were performed using NONMEM. The fit of the generalized additive model to the initial parameter estimates was performed in S-Plus (version 3.1, Statistical Sciences Inc., Seattle, WA, USA).

Results

Evaluation of cytotoxicity data

The sigmoid inhibitory effect model, including a baseline effect, described the concentration-effect profile well (Figure 2). There was no bias in fit and the distribution was equal for low and high concentrations, so the model satisfactorily described the data (data not shown). All data fulfilling the criteria, described in the section Cytotoxicity test, were included in the analysis. Population estimates of EC₅₀, Eₘₐₓ and the slope along with the relative standard error for the three parameters were estimated with non-linear mixed effect modeling. The results are presented in Table 2A. The residual error magnitude in the experiments was 12% (5-33%). The inter-individual variability, not given in Table 2A, was, in general, high for EC₅₀ with values averaging 136% (36-300%) and lower for Eₘₐₓ with values averaging 24% (7.2-41%). Using the generalized additive model, none of the covariates was found to influence the final model significantly.

Cytotoxicity of CdA and CAFdA in leukemic cells

Figure 2. Population profile for cell survival at increasing concentrations of CAFdA (●) and CdA (○) with one standard deviation presented. A sigmoidal inhibitory effect model with a baseline effect parameter best described the data.

Table 2A. Population parameter estimates, with standard errors expressed as % coefficient of variation in parenthesis.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC₅₀ (µM)</th>
<th>Eₘₐₓ</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>CdA</td>
<td>0.15 (6.9%)</td>
<td>0.79 (1.5%)</td>
<td>0.92 (9.4%)</td>
</tr>
<tr>
<td>CAFdA</td>
<td>0.12 (9.2%)</td>
<td>0.73 (1.7%)</td>
<td>1.12 (15%)</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>3.34 (33%)</td>
<td>0.74 (13%)</td>
<td>0.71 (2.2%)</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>1.95 (19%)</td>
<td>0.71 (17%)</td>
<td>1.23 (6.4%)</td>
</tr>
</tbody>
</table>

Cytotoxic activity

Using equimolar concentrations of CdA and CAFdA, the cytotoxicities (EC₅₀), as calculated with the non-linear mixed effect modeling, were in the same range for the populations, i.e. 0.15 µM for CdA and 0.12 µM for CAFdA. From the individual estimates, the EC₅₀ value for CAFdA was significantly lower than that for CdA (median EC₅₀ 0.09 µM and 0.14 µM for CAFdA and CdA respectively); the difference in cytotoxicity between CAFdA and CdA was more pronounced in CLL patients (medi-an EC₅₀ for CAFdA 0.08 µM and for CdA 0.16 µM, p<0.001), and no difference was found in AML patients (EC₅₀ for CAFdA 0.10 µM and for CdA 0.11 µM, p=0.96) (Figure 3). Using Spearman’s rank test, we found an overall significant correlation for EC₅₀ for CAFdA and Eₘₐₓ for CdA (rho=0.81, p<0.0001, Figure 4A, B) but no such correlations were found between CAFdA and fludarabine (rho=0.42, p=0.068, Figure 4C, D and Table 2B) or AraC (rho=0.27, p=0.06). The median EC₅₀ of fludarabine was substantially higher than that measured with CdA and CAFdA (Table 2A, Figure 4C, D). The reason for these differences can be explained by the fact that fludarabine is a poorer substrate for the activating enzyme, dCK, than CdA or CAFdA. However, in clinical practice these drugs were not applied at equimolar dosages.

Thirty-six (CLL n=26) of the 52 samples were more sensitive to CAFdA than to CdA. Of the remaining 16 samples, 8 were equally sensitive to both drugs. Some patients were newly diagnosed and had not been treated while others had been previously treated with a variety of drugs but only CdA in 3 cases. These 3 CLL patients were sensitive to CdA when the median EC₅₀ for CdA was used as the cut-off point but were more sensitive to CAFdA than to CdA. No significant differences were found in cytotoxicity of CdA, CAFdA, fludarabine.
and AraC between cells from refractory/relapsed and untreated AML and CLL patients.

**Enzyme activity**

The activity of dCK was analyzed in 44 and dGK in 35 of 52 patients using CdA and CAFdA. CdA is a relatively good substrate for dCK and the mitochondrial enzyme dGK was also measured using CdA phosphorylation with an excess of deoxycytidine to inhibit the dCK activity. The overall median activity for dCK was 167 (52–411) pmol/mg protein/min and the dGK activity was significantly lower, 32 (4.4–219) pmol/mg protein/min. The overall median dCK activity in CLL (n=26) was 184 (108–411) compared to 149 (52–302) pmol/mg protein/min measured in AML patients (n=18) (Figure 5). The overall median dGK activity was 35 (10–219) pmol/mg protein/min for CLL (n=21) and as low as 22 (4.4–54) pmol/mg protein/min for AML patients (n=14). However, there was no significant difference in enzyme activity between the refractory/relapsed and untreated CLL or AML patients for either dCK or dGK (Figure 5).

There was a weak correlation between the dCK and dGK activities (rho=0.34, p=0.045, n=35).

**Phosphorylation of nucleosides**

After *in vitro* incubation with 200 nM CAFdA and CdA for 2 hours, nucleotide fractions were measured.
sured in 43 of 52 patients (23 CLL and 20 AML patients) (Figures 6A-B). Overall the median level of CdAMP was 1.8 (range 0-54) µM and CdATP 1.3 (0-9.7) µM in leukemia cells. The corresponding result for CAFdAMP was 4.1 (0.7-45) µM and for CAFdATP 2.1 (0-27) µM. The levels of CAFdA nucleotides were significantly higher than the corresponding CdA levels. No significant differences were found between the formation of nucleotides in leukemia cells from patients at diagnosis and those from refractory/relapsed patients.

Figure 6. Distributions of accumulation of CAFdA and CdA monophosphates (A) and triphosphates (B) for the different diagnosed groups (▲, CLL and ▼, AML) after 2 hr of incubation with 200 nM of nucleosides. The levels of CAFdA nucleotides are significantly higher than the corresponding CdA levels. No significant differences were found between the formation of nucleotides in leukemia cells from patients at diagnosis and those from refractory/relapsed patients.

Relationship between cytotoxicity, enzyme activity and nucleoside phosphorylation

No statistically significant correlations were found in this study between in vitro cytotoxicity for nucleoside analogs to the enzyme activity or nucleoside phosphorylation, as described in the section Evaluation of cytotoxicity data. No disease specific differences between the diagnosed groups were found (data not shown). No relation was found between intracellular nucleotide concentrations, enzyme activity and the patient’s diagnosis, sex, age or previous treatment.

Discussion

Purine and pyrimidine nucleoside analogs are powerful tools in the treatment of lymphoid malignancies and some autoimmune diseases. CdA entered clinical testing in the mid-1980s and CAFdA is a new promising drug candidate with activity in hematologic malignancies2 and human colon tumor xenografts.20 CdA has a limited oral bioavailability partly due to the acid instability of the N-glycosidic linkage leading to accumulation of 2-chloroadenine. The introduction of a fluorine atom at the C-2’-arabino position of CdA resulted in a higher stability of CAFdA. The most stable compound among these analogs was found to be CAFdA.3 The metabolism of CAFdA has been previously investigated in leukemia cell lines2,21,22 and in vitro in cells from CLL and AML patients.15 In order to be active CdA, CAFdA and fludarabine must be phosphorylated by dCK and dGK. Fludarabine is a poor substrate for dCK and higher doses of fludarabine than of CdA or CAFdA must be administered.

In vitro incubation of cells from CLL and AML patients with CdA and CAFdA leads to almost double the retention of CAFdA metabolites than that of CdA metabolites, as also found by Lotfi et al.15 in a smaller set of patients. The levels of CdA intracellular phosphorylated metabolites were higher in cells from CLL patients than in those from AML patients but this difference was not found with CAFdA. This could be explained by the fact that no significant difference was found in enzyme activity between the diagnosed groups. Although, the cytotoxicity of CAFdA was significantly higher than that of CdA, the pattern of cytotoxicity was very similar for both drugs and there was a very good correlation between cytotoxicity for CAFdA levels of CdA monophosphates 1.6 (0-54) µM and triphosphates 0.7 (0-9.1) µM. The nucleotide levels of CAFdA were significant higher than those of CdA nucleotides levels except for monophosphates in AML patients. No significant differences were found in nucleotides levels between the refractory/relapsed and untreated AML or CLL patients.
and CdA. However, the majority of samples were more sensitive to CAFdA than to CdA. In a study by Parker et al., a difference in cytotoxic potency between CdA and CAFdA in CEM cells was primarily due to differences in the inhibition of ribonucleotide reductase activity rather than differences in activation by dCK or dGK. No correlation was found in this study between the level of phosphorylated metabolites and cytotoxicity.

dCK, one of the key enzymes in the intracellular metabolism of many antileukemic drugs, is well characterized and described to be a cytosolic enzyme, although data have been presented suggesting a nuclear localization. The role of dGK is less clear; the enzyme is located in the mitochondria and it has not been clarified to what extent the activity of the mitochondrial enzymes can contribute to the cytotoxicity. No previous study in cells from leukemic patients has described the levels of activating enzymes, their relation to cytotoxicity of nucleoside analogs and cellular levels of the active metabolites of nucleoside analogs. The activity of dGK was found to be lower than that of dCK, but there was a remarkable inter-individual variability for both enzymes, as also observed by previously. In patients with low dCK enzyme activity the level of dGK was not increased in compensation. In two previous studies it was found that the activity of dCK correlates with clinical responses and in a previous study we did not find a relation between clinical response and nucleotide levels. It remains unclear why enzyme activity as such, but not the levels of the active metabolite, correlates to the cytotoxic and clinical effects of CdA.

If the localization of the activating enzymes, dCK to the cytosol/nucleus and dGK to the mitochondria is considered, it is possible that the pools of active nucleoside analog metabolites are also compartmentalized. Therefore there may be major differences in mitochondrial metabolite concentrations between patients, which are not revealed when the total intracellular metabolite concentration is measured. It is possible that the subcellular localization of the intracellular metabolites of nucleoside analogs is important for their cytotoxic effects. If so, it is not relevant to try to correlate total intracellular metabolite concentration with cytotoxicity when cytosolic/nuclear and mitochondrial concentrations are not determined separately. Interestingly, a recent study showed that AraC was predominantly incorporated into nuclear DNA and arabinosylguanine into mitochondrial DNA, which suggests that the molecular targets of these nucleoside analogs may differ. Since CdA, CAFdA and fludarabine are all phosphorylated by dCK and dGK, they may target both mitochondria and nuclear DNA for their cytotoxic effects.

In this study we used non-linear mixed effects modeling to evaluate the results from the MTT assay with good results. The results from the analysis show that E_max for the different drugs did not differ much and the inter-individual variability was small (Table 2A). In contrast there was a larger distribution and inter-individual variability in the EC_{50} values. The cytotoxic effect of the used drug on the leukemia cells will vary greatly between individual patients and it is probable that the EC_{50} value will predict the efficacy of treatment when the drugs are used at a maximum tolerable dose. Apart from the given correlations, no other correlations were found between intracellular nucleotide concentrations, enzyme activity or the patient’s diagnosis, sex, age or previous treatment with the cytotoxicity for the drugs used in the study. A correlation of the cytotoxic effect between nucleoside analogs confirms previous findings while a true clinical cross-resistance is less obvious. The results from the cytotoxicity test indicated no correlation with intracellular levels of phosphorylated CdA or CAFdA. Previous studies were also unable to show a correlation between intracellular CdA metabolite accumulation in vitro and therapeutic effects in vivo. It is possible that these negative results are due to the noise created by inter-individual differences in pharmacokinetics, cytokinetics, disease stages, adverse events or social factors preventing effective therapy. Such variable noise would be eliminated in a situation in which the formation of nucleotides in vitro is compared with cytotoxicity of a fixed drug concentration also in vitro, as was the case in the present study. However, the lack of correlation between cytotoxicity and nucleotide concentration seen in this simplified experimental situation suggests that there are additional factors which determine the cytotoxic activity. Such factors could be the possible compartmentalization, as discussed above, or events beyond the bioactivation of these drugs, such as intracellular pools of endogenous nucleotides, the ability of the cells to incorporate the nucleotides into DNA, the efficacy of DNA-repair enzymes and the ability of cells to enter apoptosis. Recent studies suggest that nucleotides (CdA, CAFdA, FaraATP) can bind to apoptosis protein-activating factor 1 and co-operate with cytochrome c in order to induce caspase activation, which is important for induction of apoptosis in quiescent lymphocytes. However, another recent study demonstrated that the mechanism of resistance to CdA in cell cultures may be dictated by changes in Ca^{2+} sensitive mitochondrial events.

The leukemic cells included in this study came from patients with very heterogeneous clinical situations. Some of them were newly diagnosed and had not needed treatment while others had been...
previously treated with a variety of drugs, although CdA had been used in only 3 cases. Any correlation between the in vitro data and clinical outcome in this limited number of subjects is therefore unlikely and if present purely random. The selection of patients was aimed to provide a leukemic cell population in which to study correlations between in vitro cytotoxicity and drug metabolism, but not correlations between in vivo cytotoxicity. Thus, such in vivo/in vitro correlations were out of the scope of this study. Interestingly, cells from three patients who had previously been treated with CdA were still sensitive to CdA (when using the median EC50 for CdA as the cut-off point) but were all more sensitive to CAFdA than to CdA.

In summary we have demonstrated the higher cytotoxic activity of CAFdA compared to CdA in mononuclear cells isolated from 52 patients with CLL and AML. The greater cytotoxicity and higher intracellular metabolism of CAFdA encourage clinical trials with CAFdA in leukemic patients. Since no correlations were detected in this study between the levels of CdA and CAFdA nucleotides, dCK, dGK enzymes levels and the between the levels of CdA and CAFdA nucleotides, since no correlations were detected in this study.

References


Pre-publication Report & Outcomes of Peer Review

Contributions
All authors participated in designing the study and writing the paper. SL and YW were responsible for MTT analyses. SE was responsible for enzyme analysis and MOK contributed to the analysis and interpretation of MTT data. Primary responsibility for the paper, all Tables and Figures: SL. We thank Mats Strömberg for his skilful and reliable technical assistance and the staff at the Department of Oncology and Department of Haematology, Karolinska Hospital, for performing the blood sampling. We also like to thank Magdalena Pålsson and Tatiana Spasokouetskaja for performing some of the enzyme assays and Birgitta Pettersson for performing HPLC analysis. This work was supported by grants from the Swedish Children Cancer Foundation, Swedish Cancer Foundation and the Cancer Foundation in Stockholm.

Disclosures
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

Manuscript processing
This manuscript was peer-reviewed by two external referees and by Dr. Estella Matutes, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Matutes and the Editors. Manuscript received June 24, 2002; accepted February 6, 2003.

In the following paragraphs, Dr. Matutes summarizes the peer-review process and its outcomes.

What is already known on this topic
Cladribine and its derivative clofarabine are two purine analogs which need to be phosphorylated by kinases to be active. Cladribine has in vivo and in vitro activity in leukemic cells. Clofarabine has been shown to have in vitro cytotoxic activity in human cell lines but its use in vivo is restricted so far to phase I trials.

What this study adds
The present study demonstrates that clofarabine has a significantly higher in vitro cytotoxicity than does cladribine in cells from chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML), and that this is more pronounced in CLL. The cytotoxicity did not correlate with phosphorylation of these metabolites as assessed by measurement of enzyme activity.

Caveats
The high in vitro toxicity of clofarabine encourage the design of trials with in vivo use of this compound in leukemia patients.