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

Synnöve Lindemalm, Jan Liliemark, Astrid Gruber, Staffan Eriksson, Mats O. Karlsson, Yuying Wang, Freidoun Albertioni

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 EC_{50} 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 EC_{50} 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.

From the Dept. of Medicine, Division of Clinical Pharmacology (SL, YW, FA); Dept. of Oncology (JL); Dept. of Hematology (AG); Karolinska Hospital, Stockholm; Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala, (SE) and Dept. of Pharmacy (MOC), Uppsala University, Sweden.

Correspondence: Dr. Freidoun Albertioni, MD, Department of Medicine, Division of Clinical Pharmacology, Karolinska Hospital se-171 76 Stockholm, Sweden. E-mail: freidoun.albertioni@ks.se
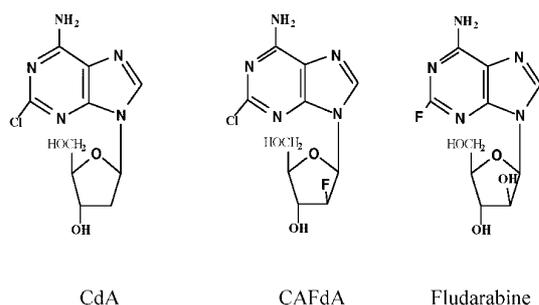
The purine analogs used in the treatment of low-grade hematologic malignancies include cladribine (2-chlorodeoxyadenosine, CdA, Leustatin®) and fludarabine (fluoroadenine-β-D-arabinoside, Fludara®) (Figure 1). Their unique characteristics in comparison with traditional antimetabolites is that they are 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 fludarabine[3] 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 disorders[5-7] and it is currently undergoing a phase II clinical trial for the treatment of pediatric refractory/relapsed acute myeloid and lymphocystic 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 CLL.[9,10] However, the formation of CdA-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

Figure 1. Structural formula of CdA, CAFdA and fludarabine.

Table 1. Characteristics of patients included in the study.

Characteristic	Total	CLL	AML
No. of patients	52	31	21
Untreated/treated	25/27	15/16	10/11
Male	29	19	10
Female	23	12	11
Age (range)	73 (22-96)	73 (46-96)	71 (22-82)
LPK (range)	56 (0.2-700)	56 (24-700)	55 (0.2-265)

Treatment data are presented as number of untreated patients in the first column and patients with previous treatment with anti-cancer drugs in the second column. Leukocyte particle concentration (LPK) is expressed as particles $\times 10^9/L$.

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 (500×g, 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 (25×10^6) 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.[14,15] 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.[16] Cells were suspended at $1-2 \times 10^6$ cells/100 µL in an extraction buffer containing 50 mM Tris-HCl pH 7.6, 2 mM dithiothreitol, 0.5 mM phenyl-methylsulfonyl fluoride, 20% glycerol and 0.5% Nonidet P40, frozen and thawed three times and centrifuged at 11,800×g 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 (DC protein assay, Bio-Rad Laboratories, CA, USA). CdA (50 μM, 10 times higher than the K_m 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 $MgCl_2$, 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 Ecoscint 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_{50}) in a MTT assay, as previously reported.[17] 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).[18] 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 interassay 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

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

Abbreviations: E=effect, E_{max}=maximal effect, E_0=baseline value, C=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_{max}, EC_{50} and the slope (γ). 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_{max} and EC_{50} were estimated. The estimates of E_{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.[19]

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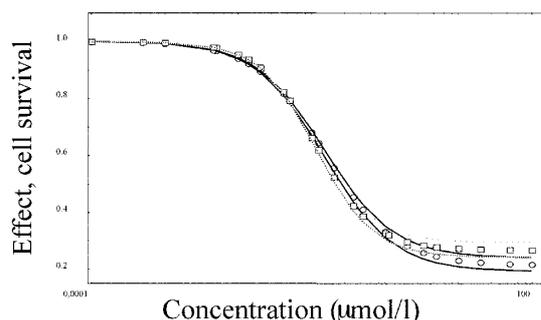
Figure 2. Population profile for cell survival at increasing concentrations of CAFdA (□) and CdA (○) with one standard deviation presented. A sigmoidal inhibitory effect model with a baseline effect parameter best described the data.

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

Drug	EC_{50} (μM)	E_{max}	slope
CdA	0.15 (6.9%)	0.79 (1.5%)	0.92 (9.4)
CAFdA	0.12 (9.2%)	0.73 (1.7%)	1.12 (15%)
Fludarabine	3.34 (33%)	0.74 (9.5%)	0.71 (9.2%)
Cytarabine	1.95 (19%)	0.71 (7.8%)	1.23 (8.4%)

Table 2B. Non-parametric correlation of individual predictions of EC_{50} values for analogs from the population analysis. A p value of less than 0.001 was considered to be statistically significant. Spearman's correlation coefficients (rho) are presented and significant results are marked with*.

	CAFdA	Fludarabine	Cytarabine
CdA	rho=0.81*	rho=0.45	rho=0.35
CAFdA		rho=0.42	rho=0.27
Fludarabine			rho=0.37

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 EC_{50}, E_{max} and the slope along 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 EC_{50} with values averaging 136% (36-300%) and lower for E_{max} with values averaging 24% (7.2-41%). Using the generalized additive model, none of the covariates was found to influence the final model significantly.

Cytotoxic activity

Using equimolar concentrations of CdA and CAFdA, the cytotoxicities (EC_{50}), 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 EC_{50} value for CAFdA was significantly lower than that for CdA (median EC_{50} 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 EC_{50} for CAFdA 0.08 μM and for CdA 0.16 μM, $p<0.001$), and no difference was found in AML patients (EC_{50} 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 EC_{50} for CAFdA and EC_{50} 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 EC_{50} 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 EC_{50} 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
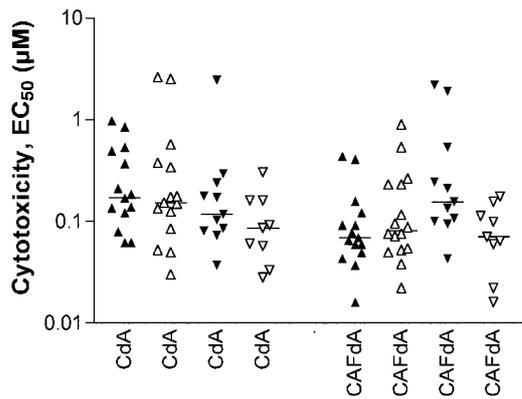
Figure 3. A scatter plot of *in vitro* response (EC$_{50}$) of leukemia cells from in CLL (▲) and AML (▼) patients using CdA and CAFdA. Open symbols correspond to refractory/relapsed patients. Median values are marked with a horizontal line. Cytotoxicity was measured by MTT assays after exposure to the various concentrations of drug for 48 hours.
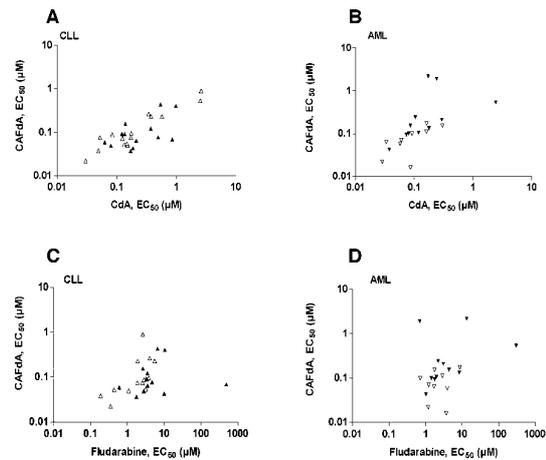

Figure 4. *In vitro* response (EC$_{50}$) for CdA (A and B) and fludarabine (C and D) are plotted versus EC$_{50}$ values for CAFdA in CLL (▲) and AML (▼) respectively. Open symbols correspond to previously treated patients. Overall a significant correlation between EC$_{50}$ for CAFdA and EC$_{50}$ for CdA (rho=0·81, *p*=0·0004) but no significant correlation was found between CAFdA and fludarabine (rho=0·42, *p*=0·068). The difference in cytotoxicity between CdA and CAFdA is highly significant in CLL but not in AML patients. No significant differences were found in cytotoxicity of leukemic cells to CdA, CAFdA and fludarabine between cells taken from patients at diagnosis and those taken from patients with refractory/relapsed disease.

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 a radioactive substrate-based assay. 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 mea-
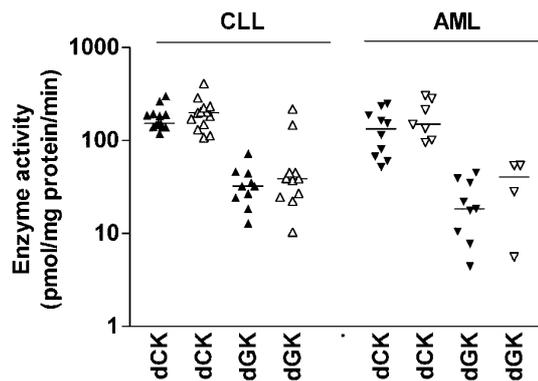

Figure 5. Levels of activating enzymes; dCK and dGK for CLL (▲) and AML (▼). Median values are marked with a horizontal line. Open symbols correspond to previously treated patients. The activity of dCK exceeds the activity of dGK. The enzyme activity varies little between the different diagnoses. No significant differences were observed between dCK or dGK activities in extracts from leukemic cells from patients at diagnosis and refractory/relapsed patients.
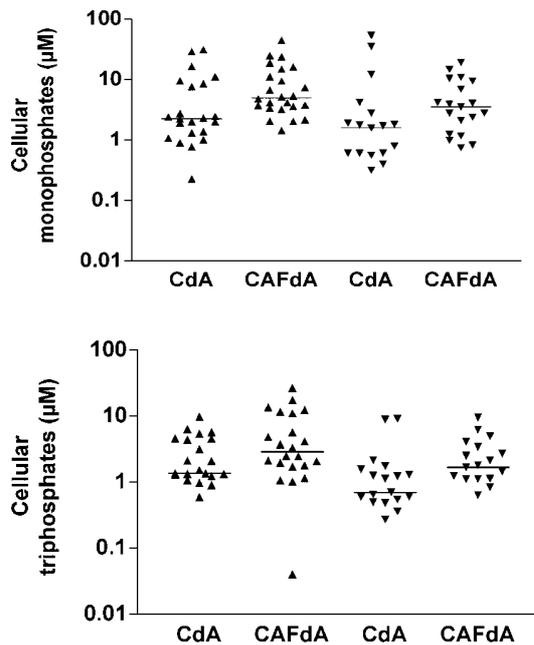
Figure 6. Distributions of accumulation of CAFdA and CdA monophosphates (A) and triphosphates (B) for the different diagnosed groups (▲, CLL and ▼, AML) after 2 hr of incubation with 200 nM of nucleosides. The levels of CAFdA nucleotides are significantly higher than the corresponding CdA levels. No significant differences were found between the formation of nucleotides in leukemic cells from patients at diagnosis and those from refractory/relapsed patients.

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 levels of CdA. The median ratio of CAFdAMP:CdAMP concentrations was 2.0 (0.2-12) and the CAFdATP:CdATP ratio was 1.6 (0-4.2). There was no significant difference in nucleotide levels between CLL and AML patients with regard to CAFdA. For CdA there was a significant difference between the diagnosed groups in levels of triphosphates and total nucleotides but not monophosphates.

In cells from CLL patients, the levels of CAFdA nucleotides were 5.0 (range 1.4-45) μM for CAFdAMP, 2.5 (0-27) μM for CAFdATP. The corresponding levels of CdA nucleotides were significantly lower, 2.2 (0-31) μM for CdAMP and 1.4 (0-9.7) μM for triphosphates. In cells from AML patients the levels of CAFdAMP were 3.5 (0.7-19) μM, CAFdATP 1.7 (0-9.5) μM and corresponding

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.

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 malignancies[2] 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 lines[2,21,22] 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

and CdA. However, the majority of samples were more sensitive to CAFdA than to CdA. In a study by Parker et al.[22] 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[23-25] although data have been presented suggesting a nuclear localization.[26] 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.[23,24,27] 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.[28] 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[9,10] and in a previous study we did not find a relation between clinical response and nucleotide levels.[13] It remains unexplained 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.[29] 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[30] while a true clinical cross-resistance is less obvious.[31] 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.[12,13] 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 (CdATP, CAFdATP, 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.[32,33] 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.[34]

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* and *in vitro* 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 EC_{50} 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 *in vitro* 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 *in vitro* cytotoxicity, it may be assumed that events beyond the phosphorylation of these nucleoside analogs are important for the final cytotoxic effect of the drugs. The biochemical and molecular bases of sensitivity to CdA and CAFdA still remain to be elucidated.

References

1. Bryson HM, Sorkin EM. Cladribine. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in haematological malignancies. Drugs 1993;46: 872-94.
2. Carson D, Wasson B, Esparza L, Carrera C, Kipps T, Cottam H. Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine. Proc Natl Acad Sci USA 1992;89:2970-4.
3. Reichelova V, Liliemark J, Albertioni F. Liquid chromatographic study of acid stability of 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, 2-chloro-2'-deoxyadenosine and related analogues. J Pharm Biomed Anal 1995;13:711-4.
4. Albertioni F, Hassan M, Silberring J, Liliemark J. Kinetics and metabolism of 2-chloro-2'-deoxyadenosine and 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine in the isolated perfused rat liver. Eur J Drug Metab Pharmacokinet 1995;20:225-32.
5. Kozuch P, Ibrahim N, Khuri F, Hoff P, Estey E, Gandhi V, et al. Phase I clinical and pharmacological study of clofarabine. Blood 1999;94:558.
6. Kantarjian H, Gandhi V, O´Brien S, Giles F, Cortes J, Kozuch P, et al. Phase I and pharmacology study of clofarabine (2-cl-2´-F-deoxy-9-d-Arabino-Furanosyladenine) in solid and hematological cancers. Blood 2001;98:4568.
7. Gandhi V, Hagop M, Kantarjian H, Faderl S, Giles F, Cortes J, et al. Cellular pharmacokinetics and pharmacodynamics of clofarabine (2-chloro-2'-fluoro-arabinosyladenine) in acute leukemia blasts during a phase I clinical trial. Proc Annu Meet Am Assoc Cancer Res. 2002:LB124.
8. Sjoberg AH, Wang L, Eriksson S. Substrate specificity of human recombinant mitochondrial deoxyguanosine kinase with cytostatic and antiviral purine and pyrimidine analogs. Mol Pharmacol 1998;53:270-3.
9. Arner ES, Spasokoukotskaja T, Juliusson G, Liliemark J, Eriksson S. Phosphorylation of 2-chlorodeoxyadenosine (CdA) in extracts of peripheral blood mononuclear cells of leukaemic patients. Br J Haematol 1994;87:715-8.
10. Kawasaki H, Carrera C, Piro L, Saven A, Kipps T, Carson D. Relationship of deoxycytidine kinase and cytoplasmic 5'-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine. Blood 1993;81:597-601.
11. Avery TL, Rehg JE, Lumm WC, Harwood FC, Santana VM, Blakley RL. Biochemical pharmacology of 2-chlorodeoxyadenosine in malignant human hematopoietic cell lines and therapeutic effects of 2- bromodeoxyadenosine in drug combinations in mice. Cancer Res 1989;49:4972-8.
12. Santana VM, Mirro J Jr, Harwood FC, Cherrie J, Schell M, Kalwinsky D, et al. A phase I clinical trial of 2-chlorodeoxyadenosine in pediatric patients with acute leukemia. J Clin Oncol 1991;9:416-22.
13. Albertioni F, Lindemalm S, Reichelova V, Pettersson B, Eriksson S, Juliusson G, et al. Pharmacokinetics of cladribine in plasma and its 5'-monophosphate and 5'-triphosphate in leukemic cells of patients with chronic lymphocytic leukemia. Clin Cancer Res 1998;4:653-8.
14. Reichelova V, Albertioni F, Liliemark J. Determination of 2-chloro-2'-deoxyadenosine nucleotides in leukemic cells by ion-pair high-performance liquid chromatography. J Chromatogr 1996;682:115-23.
15. Lotfi K, Mansson E, Spasokoukotskaja T, Pettersson B, Liliemark J, Peterson C, et al. Biochemical pharmacology and resistance to 2-chloro-2'- arabinofluoro-2'-deoxyadenosine, a novel analogue of cladribine in human leukemic cells. Clin Cancer Res 1999;5:2438-44.
16. Spasokoukotskaja T, Arner ES, Brosjo O, Gunven P, Juliusson G, Liliemark J, et al. Expression of deoxycytidine kinase and phosphorylation of 2-chlorodeoxyadenosine in human normal and tumour cells and tissues. Eur J Cancer 1995;31A: 202-8.
17. Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
18. Pieters R, Loonen A, Huismans D, Broekema G, Dirven M, Heyenbrik M, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990;76:2327-36.
19. Hastie T, Tibshirani R. Generalized additive models for medical research. Stat Methods Med Res 1995;4:187-96.
20. Takahashi T, Kanazawa J, Akinaga S, Tamaoki T, Okabe M. Antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl) adenine, a novel deoxyadenosine analog, against human colon tumor xenografts by oral administration. Cancer Chemother Pharmacol 1999;43:233-40.
21. Xie C, Plunkett W. Metabolism and actions of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-adenine in human lymphoblastoid cells. Cancer Res 1995;55:2847-52.
22. Parker W, Shaddix S, Chang C, White L, Rose L, Brockman W, et al. Effects of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine on K562 cellular metabolism and the inhibition of human ribonucleotide reductase and DNA polumerases by its 5'-triphosphate. Cancer Res 1991;51:2386-94.
23. Gower Jr WR, Carr MC, Ives DH. Deoxyguanosine kinase. Distinct molecular forms in mitochondria and cytosol. J Biol Chem 1979;254:2180-3.
24. Berk AJ, Clayton DA. A genetically distinct thymidine kinase in mammalian mitochondria. Exclusive labeling of mitochondrial deoxyribonucleic acid. J Biol Chem 1973;248:2722-9.
25. Hatzis P, Al-Madhoon AS, Jullig M, Petrakis TG, Eriksson S, Talianidis I. The intracellular localization of deoxycytidine kinase. J Biol Chem 1998;273:30239-43.
26. Johansson M, Brismar S, Karlsson A. Human deoxycytidine kinase is located in the cell nucleus. Proc Natl Acad Sci USA 1997;94:11941-5.
27. Wang L, Karlsson A, Arner E, Eriksson S. Substrate specificity of mitochondrial 2'-deoxyguanosine kinase. J Biol Chem 1993;268:22847-52.
28. Jacobsson B, Albertioni F, Eriksson S. Deoxynucleoside ana-

bolic enzyme levels in acute myelocytic leukemia and chronic lymphocytic leukemia cells. Cancer Lett. 2001;165:195-200.

29. Zhu C, Johansson M, Karlsson A. Differential incorporation of 1-β-D-arabinofuranosylcytosine and 9-β-D-arabinofuranosylguanine into nuclear and mitochondrial DNA. FEBS Lett 2000;474:129-32.

30. Larsson R, Fridborg H, Liliemark J, Csoka K, Kristensen J, de la Torre M, et al. In vitro activity of 2-chlorodeoxyadenosine (CdA) in primary cultures of human haematological and solid tumours. Eur J Cancer. 1994;30A:1022-6.

31. Juliusson G, Elmhorn-Rosenborg A, Liliemark J. Response to 2-chlorodeoxyadenosine inpatients with B-cell chronic lymphocytic leukemia resistant to fludarabine. N Engl J Med 1992;327:1056-61.

32. Genini D, Adachi S, Chao Q, Rose DW, Carrera CJ, Cottam HB, et al. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. Blood 2000; 96:3537-43.

33. Leoni LM, Chao Q, Cottam HB, Genini D, Rosenbach M, Carrera CJ, et al. Induction of an apoptotic program in cell-free extracts by 2-chloro-2'-deoxyadenosine 5'-triphosphate and cytochrome c. Proc Natl Acad Sci USA 1998;95:9567-71.

34. Chandra J, Mansson E, Gogvadze V, Kaufmann SH, Albertioni F, et al. Resistance of leukemic cells to 2-chlorodeoxyadenosine is due to a lack of calcium-dependent cytochrome c release. Blood. 2002;99:655-63.

Pre-publication Report & Outcomes of Peer Review

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.