Biochemical Pharmacology and Resistance to 2-Chloro-2′-arabino-fluoro-2′-deoxyadenosine, a Novel analogue of Cladribine in Human Leukemic Cells

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ABSTRACT

The objective of the present study was to investigate the biochemical pharmacology of 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA) — a fluorinated analogue of cladribine [2-chloro-2′-deoxyadenosine, Leustatin (CdA)] with improved acid and metabolic stability — in human leukemic cell lines and in mononuclear cells isolated from patients with chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML). We have also made and characterized two cell lines that are not sensitive to the growth inhibitory and cytotoxic effects of CAFdA. Incubation of cells isolated from the blood of CLL and AML patients with various concentrations of CdA or of CAFdA accumulated CdA and CAFdA nucleotides in a dose-dependent manner. A significantly higher rate of phosphorylation to monophosphates was observed for CAFdA than for CdA in cells from CLL patients (n = 14; P = 0.04). The differences in the phosphorylation were even more pronounced for the respective triphosphates in both CLL (n = 14; P = 0.001) and AML (n = 4; P = 0.04) cells. Retention of CAFdA 5′-triphosphate (CAFdATP) was also longer than that for CdA 5′-triphosphate (CdATP) in cells from leukemic patients. The relative efficacy of CAFdA as a substrate for purified recombinant deoxycytidine kinase (dCK), the key enzyme in the activation of nucleoside analogues, was very high and exceeded that of CdA as well as the natural substrate, deoxyctydine, by a factor of 2 and 8, respectively. The Km for CAFdA with dCK was also lower than that for CdA, as measured in crude extracts from the human acute lymphoblastic leukemia cell line CCRF-CEM and the pro-myelocytic leukemia cell line HL60. Acquired resistance to CAFdA in HL60 and in CCRF-CEM cell lines was directly correlated to the decreased activity of the nucleoside phosphorylating enzyme, dCK. Resistant cells also showed a considerable degree of cross-resistance to analogues that were activated by dCK. These observations demonstrated that dCK phosphorylates CAFdA more efficiently than CdA. Furthermore, CAFdATP is apparently more stable than CdATP and the mechanisms of resistance to CAFdA are similar to those leading to CdA resistance. These results encourage studies on the clinical effect of CAFdA in lymphoproliferative diseases.

INTRODUCTION

Progress in antileukemia chemotherapy has been achieved partially by the development of new nucleoside analogues with novel mechanisms of action such as Fara-A3 [2′F-adenine arabinoside (Fludara)] and CdA [2-chloro-2′-deoxyadenosine (Leustatin; Ref. 1)]. CdA is an effective drug for lymphoproliferative diseases, e.g., hairy cell leukemia (2, 3), CLL (4, 5), low-grade non-Hodgkin’s lymphoma (6), and AML (7, 8), as well as in autoimmune disorders and multiple sclerosis (2, 9, 10). One disadvantage of CdA is that it has only a 50% oral bioavailability (11) caused by instability at acidic pH and degradation by bacterial nucleoside phosphorylases (12).

CAFdA is a 2′-arabino-fluoro derivative of CdA. The introduction of a fluorine at the 2′-arabino position of CdA has increased its acid stability. The solubility problems associated with the administration of Fara-A and the low oral bioavailability of CdA may be overcome by using CAFdA. We have reported previously (13) that in some patients the plasma chloroadenine, a hydrolysis product of CdA, exceeded that of the CdA concentration when patients were treated with CdA p.o.

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3 The abbreviations used are: Fara-A, fludarabine; CdA, 2-chloro-2′-deoxyadenosine; CdATP, CdA 5′-triphosphate; CdAMP, CdA 5′-monophosphate; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; CAFdA, 2′-chloro-2′-arabino-fluoro-2′-deoxyadenosine; CAFdAMP, CAFdA monophosphate; CAFdATP, CAFdA triphosphate; dCK, deoxycytidine kinase; dCyd, deoxycytidine; dGK, deoxyguanosine kinase; HPLC, high-pressure liquid chromatography; AUC, area(s) under (the cellular concentration versus time) curve; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
CAFdA was more stable against deglycosylation by hepatic enzymes as compared with CdA (14).

The mechanism of action of CAFdA has been studied in detail elsewhere (15–17). CAFdA has properties that combine the features of two clinically used drugs, CdA and Fara-A. CAFdA, like CdA and Fara-A, is toxic to nondividing human lymphocytes as well as to rapidly proliferating cells. CAFdA is phosphorylated to its monophosphate mainly by dCK and also by mitochondrial enzyme dGK (18) and, after further cellular phosphorylation, to the final active metabolite CAFdATP. CAFdATP inhibited ribonucleotide reductase to an extent similar to CdATP, and DNA polymerase to an extent similar to Fara-ATP. However, data on metabolism and the mechanism of drug resistance to CAFdA are limited in humans, and, thus far, there is only one report on CAFdA pharmacokinetics in animals (19). A therapeutic effect was shown after a 1-week oral treatment with CAFdA, which eliminated 90% of the CLL cells transplanted into severe combined immunodeficiency mice (20). CAFdA administered p.o. has also been shown to be an effective agent against human colon tumor xenografts (21).

In the present study, we have characterized the intracellular metabolism of CAFdA to its mono-, di-, and triphosphate derivatives in vitro, both in human cell lines and in peripheral blood mononuclear cells isolated from CLL and AML patients. We have also determined the kinetic parameters for dCK using CAFdA as substrate in crude extracts from various human cell lines, as well as with purified recombinant dCK. The results were compared with those obtained with CdA to evaluate the therapeutic effectiveness of CAFdA as a basis for future clinical trials in humans. Moreover, human T-lymphoblastic (CRRF-CEM) and human promyelocytic leukemia (HL60) cell lines were exposed continuously to CAFdA, as a model for acquired drug resistance. The resulting cells were characterized with respect to growth inhibition, drug metabolism, cytotoxicity, and cross-resistance to other antimetabolites and clinically used anticancer drugs. These results may be important for preclinical evaluation of drug toxicity and sensitivity during CAFdA therapy.

MATERIALS AND METHODS

Leukemic Cell Isolation and Incubation. The study was approved by the ethics committee at Karolinska Hospital, Stockholm, Sweden. Blood from 14 patients with CLL and 4 patients with AML was collected from a peripheral IV access into heparinized tubes. Mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway). After washing with PBS, 2 ml of ice-cold distilled water was added to hemolyze the RBCs. After 30 s, 1 ml 2.7% NaCl was added, and the volume was adjusted to 50 ml with PBS and centrifuged. The number of cells in the samples and the median cell volume of the samples was determined by a Coulter Multisizer (Coulter Electronics, Luton, United Kingdom).

Cells were suspended in RPMI 1640 containing 25 mM HEPES (RPMI 1640-HEPES (Life Technologies, Inc., Paisley, United Kingdom) to get 1 × 10^6 cells/ml; and CdA (from Dr. Zygmunt Kazimierczuk, Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland) or CAFdA (kindly provided by Dr. Howard Cottam, University of California, San Diego, CA) was added to the medium at a concentration 0.05–25 μM. After incubation for 2 h at 37°C, cells were centrifuged, washed twice with ice-cold PBS, and extracted for nucleotide determinations, performed as described in “HPLC Determination of CdA and CAFdA Nucleotides” section. Cells from a CLL patient were incubated with 10 μM CdA or CAFdA for 2 h. After incubation, the cells were centrifuged and resuspended in drug-free RPMI 1640-HEPES. To study the efflux kinetics of CdA and CAFdA, cells were withdrawn at desired intervals, and determinations of CdA and CAFdA nucleotides were performed with HPLC as described below.

Development of Resistant Cell Lines. To delineate the mechanism of resistance to CAFdA, the human T-lymphoblastic (CRRF-CEM) and human promyelocytic leukemia (HL60) cell lines were exposed continuously to increasing concentrations of CAFdA over a period of 8–12 months. At intervals of 2 weeks, the concentrations of the drugs were increased by 5 nm. Then, the resistant cell lines were kept at concentrations of 100 nm CAFdA in CRRF-CEM cells (CEM/CAFdA) and 150 nm CAFdA in HL60 (HL60/CAFdA) cells for 2 months. The cell lines maintained stable resistance for at least 20 passages in the absence of drugs. All of the cell lines were kept in suspension culture in exponential growth in RPMI 1640 containing heat-inactivated FCS (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mm; all from Life Technologies, Inc., Paisley, United Kingdom) at 37°C in a humidified atmosphere containing 5% CO2 and were routinely tested for Mycoplasma contamination.

The number of cells in G1, S, and G2+M phase of the cell cycle were determined using a PAS II cytometer (Partec, Münster, Germany), which was equipped with a mercury arc lamp. 4,6-diamidino-2-phenylindole was excited at 365 nm and the fluorescence was measured at >435 nm. The multicycle program (Phoenix Flow Systems, San Diego, CA) was used for histogram analysis.

Before each experiment, the cell lines were subcultured in drug-free medium for at least 2 passages and then they were cultured with logarithmic growth (about 0.8–1.3 × 10^6) over a period of 3 days, washed twice with prewarmed RPMI 1640-HEPES, and counted. The cells were resuspended in the same medium to a final count of 1–2 × 10^6 cells/ml.

Cytotoxicity Assay. The cytotoxicity assay was performed as described by Mosmann et al. (22). Briefly, cell lines were suspended at 2 × 10^5 cells/ml, and 100-μl aliquots were dispensed into 96-well round-bottomed microtiter plates that already contained 5 μl of drug dilutions in triplicate. Wells containing no drugs were used as controls, and wells containing no cells were used as blanks. The plates were then incubated at 37°C for 72 h in an atmosphere of 5% CO2. We also investigated whether a continuous or a short incubation of CAFdA might have different efficacies in CCRF-CEM and HL60 cells. The cells were incubated either with 50 nm drug continuously for 120 h or with 300 nm drug for 2 h every 24 h over five days. Afterward, 10 μl of a stock solution of 5 mg/ml MTT (Sigma, St. Louis, MO) was added to each well, and the plates were incubated for an additional 4 h at 37°C. Formazan crystals were dissolved with 100 μl of 10% SDS/10 mM HCl solution overnight at 37°C. Absorbance (A) was measured using an ELISA
plate reader (Labsystems Multiscan RC) at the wavelength of 540 nm with reference at 650 nm. Cell survival (CS) was expressed as percentage of control wells:

\[
CS = \frac{\text{mean } A_{\text{treated wells}}}{\text{mean } A_{\text{control wells}}} \times 100\%
\]

**HPLC Determination of CdA and CAFdA Nucleotides.** Intracellular CdA and CAFdA nucleotides were determined as described previously (23). Briefly, 200 μl of ice-cold 0.4 M perchloric acid containing 

**Measurement of Activities of Deoxynucleoside Kinases in Crude Extracts and Determination of the Kinetic Parameters of Purified Recombinant Enzyme.** dCK activities were measured in crude extracts according to a procedure described previously by Spasokoukotskaja et al., (24). The pure recombinant dCK was prepared as described elsewhere (25). Briefly, cells were suspended to 10⁶ cells/ml in an extraction buffer containing 50 mM Tris-HCl (pH 7.6), 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.5% NP40. The suspended cells were then washed as described by Spasokoukotskaja et al. (24). The pure recombinant dCK was prepared as described elsewhere (25). Briefly, cells were suspended to 10⁶ cells/ml in an extraction buffer containing 50 mM Tris-HCl (pH 7.6), 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 0.5% NP40. The suspended cells were then washed as described by Spasokoukotskaja et al. (24). Michaelis-Menten constant (Km) and maximum velocity (Vmax) were determined over various CdA concentrations. The specific activities of the enzymes were expressed as pmol of nucleoside phosphorylating that substrate. The activities were initiated by the addition of 2–3 μg protein to a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 5 mM ATP, 4 mM DTT, 10 mM sodium fluoride, and substrates in a total volume of 25 μl. After incubation at 37°C for 15 and 30 min., 10-μl aliquots were withdrawn and spotted on WHATMAN DE-81 filter discs, which were then washed as described by Spasokoukotskaja et al. (24). Michaelis-Menten constant (Km) and maximum velocity (Vmax) were determined over various CdA concentrations. The specific activities of the enzymes were expressed as pmol of nucleoside phosphorylated by extract material from 10⁶ cells over 1 min.

Measurement of dCK activity with purified enzyme with dCyd, CdA, and CAFdA as substrates was performed using the phosphoryl transfer assay containing 100 μM [³²P]ATP (10 μCi/μl), 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 125 mM KCl,
Besides this, in AML patients, the triphosphate:monophosphate
PdATP concentrations considerably exceeded those of CdATP at
accumulation showed practically no differences, whereas CAF-
AML patients, the kinetic of CAFdA and CdA monophosphates
analysis and between CLL and AML patients by unpaired
significant.

The differences between CdA and CAFdA nucleotides were analyzed by paired
(26; DC protein assay, Bio-Rad Laboratories, CA). The differences between
moleculs concentration were calculated according to the linear trap-
tide concentration were calculated according to the linear trap-

RESULTS

Comparison of Cellular Accumulation and Retention of
CAFdA and CdA Metabolites in Leukemic Cells. Analysis
of the nucleotide fractions by HPLC after in vitro incubation of
leukemic cells from CLL (n = 14) and AML (n = 4) patients for
2 h, with increasing concentrations of CdA and CAFdA, showed a concentration-dependent accumulation of CdA and CAFdA
nucleotides (Fig. 1, A and B).

In cells from CLL patients, the initial and maximal velocities of CAFdAMP and CAFdATP accumulation were significantly
elevated compare to those for CdAMP and CdATP (Fig.
1A). In CLL cells at 1-µM exogenous concentration of ana-
logues, the mean ratio of CAFdAMP:CdAMP concentrations was 1.6 ± 0.5 (P = 0.05), and an even higher CAFdATP:
CdATP ratio was achieved (4.3 ± 3.5; P = 0.001). In cells from
AML patients, the kinetic of CAFdA and CdA monophosphates
accumulation showed practically no differences, whereas CAF-
daTAP concentrations considerably exceeded those of CdATP at
all of the concentrations of analogues (P = 0.04; Fig. 1B).

Besides this, in AML patients, the triphosphate:monophosphate
ratios, as well as the absolute triphosphate concentrations of
both analogues were 3- to 4-fold higher compared with CLL
patients (Fig. 1, A and B).

The retention of CAFdA and CdA nucleotides accumulated
in CLL cells after a 2-h exposure to 10-µM concentrations of
analogs was followed up to 48 h after the exposure. The decay
of CAFdA mono- and triphosphates in cells after resuspension
in drug-free medium was biphasic with a prolonged terminal
phase for CAFdATP. The intracellular half-life of CAFdATP
was longer than that for CdATP (t1/2: 7.3 and 4.3 h, respective-
ly). The CAFdAMP exposure, expressed as the area under the
nucleotide concentration versus time curve (AUC0–48), to the
CLL cells was higher for CAFdA (302 ± 340 µM × h) when compared
with corresponding values of CdA (117 ± 14 µM × h). The differences
in the AUC for triphosphates were even more pronounced
(250 and 66 µM × h for CAFdATP and CdATP, respectively).
More than 90% of the CdATP and CAFdATP was eliminated

Table 1  Kinetic parameters of nucleoside analogues with dCK by
purified recombinant dCK using phosphoryl transfer assay

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Vmax</th>
<th>Relative efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified dCK</td>
<td>dCyd</td>
<td>6.3 ± 2.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>CdA</td>
<td>5.8 ± 0.9</td>
<td>2.1 ± 0.1</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>CAFdA</td>
<td>3.3 ± 0.6</td>
<td>4.0 ± 1.0</td>
<td>760</td>
</tr>
</tbody>
</table>

" Data are expressed as mean ± SD of at least two separate experiments performed in triplicate.

10 mM DTT, 0.5 mg/ml BSA, dCK, and various concentrations of
nucleoside in a total volume of 50 µl. The phosphorylated products were separated by TLC and quantitated as described
previously (26). Vmax and Km were calculated using a commer-
cially available program, GraphPad PRISM Version 2.0 (Intui-
tive Software for Science, San Diego, CA).

Western Blotting. The amount of dCK protein was
determined in HL60 and CCRF-CEM cell extracts by Western
blot. A peptide anti-dCK antibody was used as previously de-
scribed (27). Approximately 40 µg of crude cell extract and 2
µg of purified recombinant dCK were analyzed, and the protein
bands were detected by the enhanced chemiluminescence
method as described by the suppliers (Amersham).

Protein Determination. The protein content in cell ex-
tracts was determined according to the method of Lowry et al.
(28; DC protein assay, Bio-Rad Laboratories, CA).

Kinetic and Statistical Calculations. The areas under the
cellular concentration versus time curve (AUC) from time 0
to the time that the last measured CdA and CAFdA nucleo-
tides concentration were calculated according to the linear trap-
zoidal rule. Statistics were calculated using StatView software
(Abacus Concepts, Berkeley, CA). The differences between
CdA and CAFdA nucleotides were analyzed by paired t test
analysis and between CLL and AML patients by unpaired t test
analysis. A P of less than 0.05 was considered to be statistically
significant.

Fig. 2 Comparative toxicity of CdA (●) and CAFdA (○) toward (A)
CCRF-CEM/wt (···) and the corresponding CAFdA resistant cell lines
(---), and toward (B) HL60/wt (···) and the corresponding CAFdA
resistant cell lines (——). Cells were suspended at a cell density of
0.2–0.3 × 10⁶/ml in medium in microtiter plates containing various
concentrations of CdA or CAFdA and incubated at 37°C for 72 h. The
viability was assessed with MTT assay as specified in “Materials and
Methods.” The data were presented as mean of at least 2 separate
experiments performed in triplicate.
from the cell within 48 h of incubation in drug-free medium (data not shown).

**Phosphorylation of CAFdA and CdA by Pure Recombinant dCK and by the Kinases in Crude Cell Extracts.** An explanation for the higher intracellular levels of CAFdATP can be found in the more efficient phosphorylation of CAFdA by dCK as compared with CdA. Table 1 shows the $K_m$ and relative $V_{\text{max}}$ values for CdA, CAFdA, and natural substrate, dCyd, with recombinant dCK using the phosphoryl transfer assay. CAFdA was three times more efficient than CdA and showed nearly 8-fold higher efficiency than the natural substrate dCyd. In crude extracts of CCRF-CEM cells, the $K_m$ values—measured with radiolabeled dCyd, CdA, and CAFdA—were 2.5, 3.4, and 2.3 $\mu$M, respectively. In extracts of HL60 cells, the $K_m$ values were 2.0, 2.9, and 1.4 $\mu$M, respectively. Thus both the recombinant dCK and the enzyme from leukemic cells had higher affinity to CAFdA, than to CdA.

**Sensitivity and Resistance to CAFdA in CCRF-CEM and HL60 Cells.** Human leukemia cell lines growing in suspension cultures were incubated with different concentrations of CAFdA. The concentrations of analogue-inhibiting cell growth by 50% ($IC_{50}$) in CCRF-CEM and HL60 were 0.05 ± 0.02 $\mu$M and 0.04 ± 0.00 $\mu$M, respectively, as measured after 72 h (Fig. 2, A and B). We found a similar toxicity toward CCRF-CEM and HL60 when cells were exposed to CAFdA as continuous treatment (50 nm for 120 h) or short treatment (300 nm for 2 h every 24 h for 5 days; data not shown). The choice of CAFdA concentration was based upon the concentration achievable clinically in plasma in patients treated with CdA as continuous infusion (steady state level) or as short infusions (maximum concentration), respectively (11).

To investigate the molecular mechanisms of resistance to CAFdA, CCRF-CEM and HL60 cells were exposed to CAFdA continuously at concentrations achievable clinically in patients treated with CdA (10–300 nm). Two resistant stable clones designated as CEM/CAFdA and HL60/CAFdA were selected and characterized with respect to growth inhibition and cross-resistance to other antimetabolites and clinically used anticancer drugs. The cells were more than 1000-fold less sensitive to the growth inhibitory and cytotoxic effects of CAFdA relative to
wild-type (Fig. 2; Table 2). Resistant cells also showed a considerable degree of cross-resistance to analogues such as Cda, Fara-A, 1-β-d-arabinofuranosylcytosine, difluorodeoxyguanosine, and difluorodeoxycytidine (data not shown). We found no differences in the sensitivity of resistant cells to tubercidin, vincristine, daunorubicin, idarubicin, mitoxantrone, 5-fluorouracil, or paclitaxel (data not shown). The percentage of cells in S phase and the thymidine kinase activities did not differ substantially between the CAFdA-resistant cells and wild-type cells (Table 2). Resistant cell lines accumulated lower levels of CAFdA nucleotides (Table 2) as well as Cda metabolites (data not shown). To investigate the mechanisms for this reduced anabolism, we measured the dCK activities using dCyd as substrate in the four cell lines. CEM/CAFdA and HL60/CAFdA had 98 and 76% reduced dCK activity, respectively, as compared with the wild-type cells (Table 2). The dCK activity measured with Cda as substrate was slightly higher than for the natural substrate, dCyd, probably because the latter is also a good substrate for the mitochondrial enzyme, dGK but showed a similar decrease. The dCK protein levels were also determined using immunoblotting. There was no detectable dCK in the CAFdA-resistant cell line extracts, whereas it was easily measured in CEM and HL60 wild-type cell extracts (Fig. 3). Thus, the protein levels appeared to be proportional to the dCK activity measurements in these cells. The relative activity of dGK (an enzyme that also phosphorylates Cda and CAFdA) was not changed in HL60/CAFdA cells, but it was slightly elevated in CEM/CAFdA (Table 2).

DISCUSSION

Cda and Fara-A have been proven to be effective in the treatment of hematological malignancies. CAFdA, a novel analogue of Cda and Fara-A, was synthesized about 10 years ago, but it still has not entered clinical evaluation. Increased knowledge of CAFdA metabolism could be valuable for future clinical use of CAFdA. In vitro cell culture studies and animal studies indicate that CAFdA is an effective drug that may have benefit in the treatment of blood malignancies (20). The metabolism of CAFdA has been previously investigated in leukemic cell lines (16, 20), but no study in patient cells, except from the present report, describe the metabolism of CAFdA and compare it to Cda in cells from leukemic patients. In vitro incubation of mononuclear cells from CLL and AML patients with identical exogenous drug concentrations leads to a higher intracellular accumulation of CAFdATP compared with CdaATP. In these cells, the major metabolite was CAFdAMP, and the accumulation of CAFdADP represented less than 10% of the total nucleotides, indicating that dCK is not the rate-limiting step in the generation of CAFdATP, as has been shown for Fara-A, arabinofuranosylcytosine, and difluorodeoxycytidine. However, we found several differences in the metabolism of Cda and CAFdA in human cell lines. The retention of CAFdA metabolites were higher compared with Cda metabolites. The 48-h concentration of CAFdATP was more than 2-fold higher than the concentration measured for CdaATP. These results indicate that intermittent treatment with CAFdA may result in a higher level of CAFdATP as compared with Cda treatment and CdaATP levels. The more efficient phosphorylation of CAFdA in leukemic cells from patients at low nucleoside concentrations is most likely due to the fact that CAFdA is a better substrate for dCK than Cda. This was observed both with pure recombinant dCK and with enzyme measurement of endogenous dCK in crude cell extracts.

Initial studies with CAFdA indicated that mutant lymphoblasts with reduced dCK activity and with elevated cytoplasmic 5'-nucleotidase, or with expanded dNTP pools, were resistant to CAFdA toxicity (20). However, the mechanism of acquired resistance to CAFdA has not been investigated thus far. In an attempt to achieve this goal, we developed CAFdA-resistant CCRF-CEM and HL60 cells. We found no cross-resistance or changes in sensitivity to a marker for nucleoside transport, tubercidin, or multidrug resistance-related drugs, like vincristine, daunorubicin, idarubicin and paclitaxel or to other types of drugs such as 5-fluorouracil. This indicates that the mechanism of acquired resistance to CAFdA is specific and not a general phenomenon. The CAFdA-resistant cell lines accumulated less CAFdA nucleotides and had a lower levels of the phosphorylating enzyme dCK as well as lower dCK protein than the wild-type cells (Table 2; Fig. 3). Recent experiments showed that the efficiency of CAFdA as substrate for the mitochondrial enzyme dGK was similar to Cda and it was 60% of that of the natural substrate, deoxyguanosine (18). In cells and tissues where dCK is absent, dGK is most likely to be the main Cda- and CAFdA-activating enzyme. It is notable that some increase in the relative activity of dGK was observed in the CCRF-CEM mutant cell line (Table 2). These cells were shown to be more sensitive to Cda than to CAFdA (Fig. 2A). Thus, dGK may have a significant role in the cytotoxicity of nucleoside analogues in leukemic cells lacking dCK (29).

Taken together, the mechanisms of acquired resistance to CAFdA are similar to those leading to Cda resistance. Compared with Cda, CAFdA is phosphorylated more efficiently and eliminated more slowly, leading to differences in the cellular pharmacokinetics and pharmacology of the two drugs. On the basis of these results, one would expect not only greater antitumor effects but also higher hematological toxicity of CAFdA compared with Cda because dCK is constitutively and highly expressed in bone marrow and normal lymphocytic cells (24). Moreover, the substitution of hydrogen by fluorine at the C-2′-arabino (up) position in CAFdA not only increased the chemical stability of the glycosidic bond of the compound to acidic or enzymatic hydrolysis but also resulted in a higher lipophilicity of the nucleoside (30). This would favor its penetration through the blood-brain barrier into the brain, in which the expression of dCK is low but the activity of dGK is about 10-fold higher than in other tissues (25). Surprisingly, in mice, the concentration of CAFdA in the brain was found to be lower than that of Cda (31). Whether CAFdA will be an effective drug against lymphoproliferative diseases remains to be investigated.

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REFERENCES


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