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Down-regulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance

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Abstract

Mechanisms of acquired resistance to three purine analogues, 2-chloro-2′-deoxyadenosine (cladribine, CdA), 9-β-D-*arabino*furanosyl-2-fluoroadenine (fludarabine, Fara-A), and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (clofarabine, CAFdA) were investigated in a human T-lymphoblastic leukemia cell line (CCRF-CEM). These analogues are pro-drugs and must be activated by deoxycytidine kinase (dCK). The CdA and CAFdA resistant cell lines exhibited increased resistance to the other nucleoside analogues activated by dCK. This was also the case for the Fara-A resistant cells, except that they were sensitive to CAFdA and guanosine analogues. The CdA and CAFdA resistant cells displayed a deficiency in dCK activity (to <5%) while the Fara-A resistant cells showed only a minor reduction of dCK activity (20% reduction). The activity of high K_m 5′-nucleotidase (5′-NT) (cN-II) using IMP as substrate, was 2-fold elevated in the resistant cell lines. The amount of the small subunit R2 of ribonucleotide reductase (RR) was higher in the Fara-A resistant cells, which translated into a higher RR activity, while CdA and CAFdA cells had decreased activity compared to the parental cells. Expression of the recently identified RR subunit, p53R2 full-size protein, in CAFdA cells was low compared to parental cells, but a protein of lower molecular weight was detected in CdA and CAFdA cells. Co-incubation of Fara-A with the RR inhibitor 3,4-dihydroxybenzohydroxamic acid (didox) enhanced cytotoxicity in the Fara-A resistant cells by a factors of 20. Exposure of the cells to the nucleoside analogues studied here also caused structural and numerical instability of the chromosomes; the most profound changes were recorded for CAFdA cells, as demonstrated by SKY and CGH analysis. We conclude that down-regulation of dCK in cells resistant to CdA and CAFdA and increased activity of RR in cells resistant to Fara-A contribute to resistance.

Keywords: Cladribine; Clofarabine; Fludarabine; Ribonucleotide reductase; Deoxycytidine kinase

1. Introduction

Purine nucleosides are important agents in the treatment of hematological malignancies. 2-Chloro-2′-deoxyadenosine (cladribine, CdA) and 9-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine, Fara-A) have been used successfully in the treatment of chronic lymphocytic leukemia and low-grade lymphoma [1,2]. CdA is particularly effective against hairy cell leukemia with a >90% response rate. 2-Chloro-2′-*arabino*-fluoro-2′-deoxyadenosine (clofarabine, CAFdA) is a new 2′-*arabino*-fluoro derivative of CdA (Fig. 1), which

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Abbreviations: CdA, 2-chloro-2′-deoxyadenosine; Fara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; CAFdA, 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine; dCK, deoxycytidine kinase; 5′-NT, 5′-nucleotidase; dGK, deoxyguanosine kinase; RR, ribonucleotide reductase; dNTP, deoxyribonucleotide triphosphates; HU, hydroxyurea; didox, 3,4-dihydroxybenzohydroxamic acid; Ara-C, cytosine arbinoside; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; dFdC, difluorodeoxycytidine; dFdG, difluorodeoxyguanosine; P-gp, P-glycoprotein; MRP, multidrug resistance associated protein; SKY, spectral karyotyping; CGH, comparative genomic hybridization.

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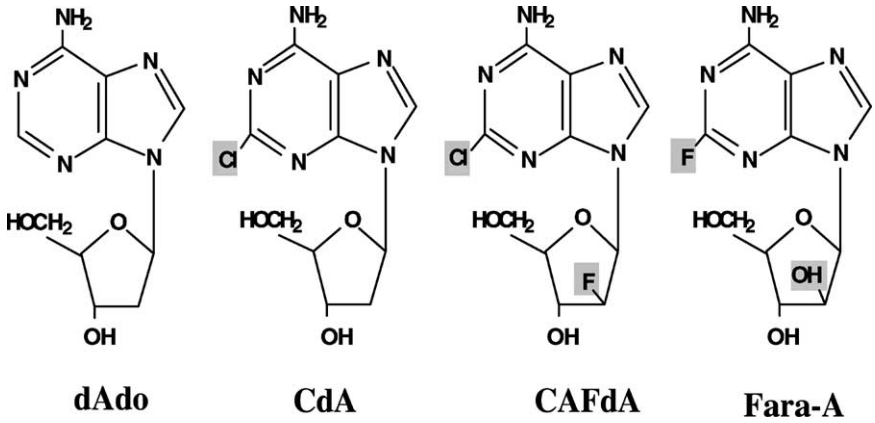

Fig. 1. Structures of deoxyadenosine, CdA, CAFdA and Fara-A. Substitution with a chloro or fluoro atom at the 2-position of the adenine ring makes the compounds resistant to deamination by adenosine deaminase. At the 2′-arabino position, CAFdA has a fluoro atom and Fara-A has a hydroxy group.

has been used in a phase I clinical trial with encouraging preliminary results [3] and is currently undergoing phase II clinical trial for pediatric refractory/relapsed acute myeloid and lymphoblastic leukemia. We recently reported that CAFdA is a more efficient substrate for the activating enzyme and that the active form is more stable, due to higher phosphorylation and longer retention time compared to CdA, and that the mechanisms leading to acquired resistance to CAFdA are similar to those for CdA in human leukemia cell lines [4].

Nucleoside analogues are pro-drugs that require activation by phosphorylation for cytotoxicity. Deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) catalyze the initial phosphorylation [5], and 5′-nucleotidases (5′-NT) oppose the activity of dCK. Resistance to nucleoside analogues has been shown to be caused by dCK deficiency in different cell lines [4,6,7]. Ribonucleotide reductase (RR) converts the nucleoside diphosphates to the corresponding deoxynucleoside diphosphates [8]. RR consists of two subunits, R1 and R2, which are both required for activity. The specificity and the overall activity of RR is controlled by binding ATP and dNTP allosteric effectors to a specificity site and an activity site on the R1 subunit [8]. CdA, CAFdA and Fara-A target RR, resulting in alteration of the deoxynucleoside triphosphates generated in large amounts by RR that may compete with nucleotide analogues for incorporation into DNA. RR may also be insensitive to allosteric regulation due to the mutations or altered regulation of the enzyme.

One rationale to overcome resistance caused by large amounts of dNTP pools is the use of RR inhibitors, i.e. hydroxyurea (HU) the iron chelator and the radical scavenger 3,4-dihydroxybenzohydroxamic acid (didox). At the present time, HU is the only clinically used RR inhibitor [9]. Didox is a polyhydroxysubstituted benzohydroxamate, which has been demonstrated to be a potent inhibitor of RR [10]. HU used in combination with cytosine arabinoside (Ara-C) in the Ara-C-resistant human promyelocytic leukemia cell line HL60 enhanced the cytotoxic effect of Ara-C [11].

Drug resistance jeopardizes cancer treatment in general and nucleoside analogue therapy is no exception. Detailed knowledge of the molecular mechanisms underlying resistance would enable the use of appropriate combination therapies in the clinical setting. In the present study we report a comparison of the mechanism of resistance to CdA, CAFdA and Fara-A in CCRF-CEM leukemic cells grown in culture. We investigated key enzymes in the metabolism of nucleoside analogues, to which we detected differences in the pattern of resistance.

2. Methods

2.1. Chemicals

CdA was synthesized by Dr. Zygmunt Kazimierczuk, Foundation for Development of Diagnostics and Therapy. Fara-A was a gift from Dr. Ze'ev Shaked (Berlex, Alameda). CAFdA was a gift from Howard Cottam at the University of California. Deoxyadenosine, Ara-C, hydroxyurea, tubercidin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), ammonium dihydrogenphopshate ($NH_4H_2PO_4$), dithiothreitol, phenylmethylsulfonyl fluoride, Nonidet P-40, α,β-methylene adenosine 5′-diphosphate, inosine, imidazole, hypoxanthine, inosine 5′-monophosphate, β-glycerophosphate, adenosine 5′-triphosphate and ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Sigma. Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was purchased from Burroughs Wellcome Co. and SDS was purchased from KEBO Lab. Difluorodeoxycytidine (dFdC) and difluorodeoxyguanosine (dFdG) was obtained from Lilly. Didox (3,4-dihydroxybenzohydroxamic acid) was from Molecules for Health Inc. 9-β-D-Arabinofuranosylguanine Ara-G was from RI Chemical. Daunorubicin was purchased from Rhone-Poulenc Rorer. 5-^{3}H-dCyd (16.7 Ci/mmol), 8-^{3}H-Ara-G, 8-^{14}C-inosine 5′-monophosphate were purchased from Moravek Biochemicals. 5-^{3}H-Ara-C and 5-^{3}H-CDP were obtained

from Amersham. CdATP was synthesized by Sierra Biosearch. RPMI 1640 medium, heat-inactivated fetal calf serum, L-glutamine and penicillin–streptomycin were from Gibco (Life Technologies). All reagents for PCR reactions were purchased from Perkin-Elmer.

2.2. Cell specimens

The CCRF-CEM human T-cell line was obtained from American Type Culture Collection. The CCRF-CEM cell line was made resistant to CdA, CAFdA and Fara-A by exposure to stepwise increasing concentrations. Briefly, at intervals of 2 weeks the concentrations of the drugs were increased by 5 nM until concentrations of 100 nM CdA and CAFdA and of 300 nM Fara-A were reached. Then the drug concentrations were increased by 10 nM for CdA and CAFdA and 50 nM for Fara-A until the final concentrations of 1 μM were tolerated by the respective cell lines. The cell lines maintained stable resistance in the absence of drug for at least 20 passages. Single clones were isolated by limiting dilutions. Cells were grown in RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) at 37° in a humidified air atmosphere containing 5% CO_2 and were routinely tested for mycoplasma contamination. The number of cells in the samples and the median cell volume of the samples were determined using a Coulter Multisizer (Coulter Electronics). Before conducting any experiments the cells were cultured in drug-free medium for three passages.

2.3. MTT chemosensitivity assay

Cells were seeded at a concentration of 2×10^4 cells per well in 96-well microtiter plates containing various concentrations of drugs in triplicate and plates were incubated at 37° for 72 hr in supplemented RPMI 1640 medium. To measure the cytotoxicity to dAdo, cells were preincubated with 10 μM EHNA for 1 hr prior to the addition of dAdo and during the 72 hr incubation to inhibit degradation by adenosine deaminase. Following incubation, 10 μL MTT solution (5 mg/mL tetrazolium salt) was added to each well and the plates were further incubated for 4 hr. The formazan salt crystals were dissolved in 100 μL 10% SDS in 10 mM HCl solution overnight at 37°. The optical density (OD) of the wells was measured at a wavelength of 540 nm with reference of 650 nm using an ELISA plate reader (Labsystems Multiscan RC). Cell survival in a well was expressed as percentage compared to growth in control wells.

2.4. Drug accumulation studies

In order to study the accumulation of CdA, CAFdA, Ara-C and Fara-A nucleotides in resistant cells, exponentially growing cells were exposed to 1 μM CdA and CAFdA, 5 μM Ara-C or 30 μM Fara-A for 2 hr. The cells were centrifuged at 1200 *g* for 5 min, washed twice with cold PBS and the intracellular accumulation of nucleotides were analyzed. CdA and CAFdA nucleotides were extracted and analyzed according to Reichelova *et al.* [12]. For analysis of Ara-C and Fara-A nucleotides, cells were extracted with 0.4 M perchloric acid and neutralized with 1.2 M KOH/0.5 M $NH_4H_2PO_4$, centrifuged and the supernatant was collected. Ara-CTP and Fara-ATP were separated from endogenous nucleotides using a Partisil-10 SAX anion exchange column (250 mm × 4 mm, Whatman), with gradient elution [13]. The concentration (expressed in μM/hr) was calculated by dividing the total amount of each nucleotide in the sample by the number of cells and the mean cell volume of the sample per hour.

2.5. Measurement of dCK, dGK, thymidine kinase and 5′-NT activities

Cells were suspended at 10^6 cells/100 μL in an extraction buffer containing 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% Nonidet P-40 and were freeze-thawed three times. The cell extracts were centrifuged at 11,800 *g* in an Eppendorf centrifuge for 5 min at 4° and the supernatants were stored at −20° until use. Enzyme activity was measured radiochemically according to a method previously described by Spasokoukotskaja *et al.* [14]. Substrates were deoxycytidine (10 μM), thymidine (10 μM) and Ara-G (100 μM). To measure dGK activity, Ara-G was used as substrate and excess of deoxycytidine (1 mM) was added. The assays were initiated by addition of 2–3 μg protein into 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 in a total volume of 25 μL. After incubation at 37° for 20 min, 10 μL aliquots were withdrawn and spotted on WATMAN DE81 papers, which were then washed before reading in a liquid scintillation counter. The activities were expressed as pmol/mg cellular protein/min.

The activity of 5′-NT was measured using a radiochemical assay previously described by Spychala and Mitchell [15]. The reaction mixture had a total volume of 25 μL and contained 50 mM imidazole, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 g/L BSA, 0.2 mM α,β-methylene adenosine diphosphate (which inhibits the activity of the membrane-bound 5′-ecto-NT), 5 mM β-glycerophosphate, 100 μM EGTA and 50–200 μM IMP and [^{14}C]-IMP as substrate. To measure high-K_m 5′-NT, 3 mM ATP is added to activate this enzyme. The reaction was assayed for 30 min at 37° and stopped by adding formic acid, then briefly centrifuged and 10 μL was added to a thin-layer chromatography plate (PEI-cellulose F). The plate was developed in 1-butanol:-H_2O:methanol:NH_4 (60:20:20:1 v/v). Spots that had co-migrated with the inosine and hypoxanthine markers were visible in UV-light and cut out after which 3 mL of Ultima

Gold (Packard) was added. The radioactivity was counted in a liquid scintillation counter (RackBeta, LKB Wallac). The enzyme activity was expressed in picomoles per minute per mg of protein.

2.6. RNA extraction and real-time quantitative PCR

Cells were collected by centrifugation and RNA extraction, cDNA synthesis and real-time quantitative PCR were performed as previously described [16]. Primers for dCK, dGK, 5′-NT and the internal control, β2-microglobulin were described earlier [16]. Reactions were performed and data collected using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Each assay included cloned cDNA standards in triplicate, nontemplate controls and the unknown samples in triplicate. Results are expressed as the ratio of dCK, dGK or 5′-NT to β2-microglobulin.

2.7. Sequencing of dCK

In order to clone and sequence the dCK gene the pCR®II-TOPO® vector (Invitrogen) was used. The dCK gene was amplified by PCR of cDNA from the CEM cells using as forward primer; 5′-ACACCATGGCCACCCCG-CCCAAGAGAAGCT-3′ and as reverse primer; 5′-CAC-GGATCCTCACAAAGTACTCAAAAACTCTTT-3′. The PCR products were ligated into the pCR®II-TOPO® vector, and the ligated material was further transformed into TOP10 *E. coli* cells by chemical transformation. Cells carrying recombinant plasmids were screened for white colonies on LB agar plates containing ampicillin (50 μg/mL), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactoside (IPTG). The clones were analyzed for fragments with the appropriate size by PCR using the following primers; M13 (−20) forward, 5′-GTAAAACGACGGCCAG-3′ and M13 reverse, 5′-CAGGAAACAGCTATGAC-3′. The sized PCR products were sequenced according to the manufacturers' instructions (PRISM Big Dye Ready Reaction DyeDeoxy Terminator sequencing kit (Perkin-Elmer)) and analyzed using an automatic DNA capillary sequencing apparatus ABI 310 kit (Perkin-Elmer).

2.8. SDS–PAGE and Western blotting

Cells were centrifuged and washed twice with PBS. The pellets were suspended in lysis buffer containing protease inhibitors (Complete Mini tablets; Boehringer-Mannheim) and sonicated for 3 × 10 s. For determination of P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP1–6) levels, membrane protein extracts were prepared as previously described [17]. Polypeptides were resolved in 4–15% SDS–polyacrylamide gels (Bio-Rad Laboratories) run at 120 V and electrophoretically transferred to polyvinylidene difluoride membranes for 1 hr at 60 V. Membranes were blocked overnight in a TBST buffer (25 mM Tris, pH 8.0, 150 mM NaCl and 0.5% Tween-20) containing 5% (w/v) nonfat dried milk. Specific antibodies for dCK [18] and dGK [19] were used. A monoclonal antibody (ADα03) was used to detect ribonucleotide reductase R1 subunit (InRo Biomedtek) and the polyclonal antibody used to detect R2 was kindly provided by Dr. David Lembo at the Department of Public Health and Microbiology, University of Turin and prepared as described [20]. The polyclonal antibody for p53R2, ab8105 was purchased from Abcam. Blots were probed for 1–2 hr with primary antibody for dCK (1:10,000), dGK (1:600), R1 (1:1000), R2 (1:10,000), p53R2 (1:1000) and MRP1–6 (Santa Cruz Biotechnology Inc., diluted 1:1000), and P-gp (Accurate Chemical & Scientific, diluted 1:1000). Blots were then probed with horseradish peroxidase-coupled antirabbit secondary antibody for dCK, dGK, R2, p53R2, P-gp, an antimouse secondary antibody for R1 or an antigoat secondary antibody for MRP1–6. The immunoreactive bands were visualized using the enhanced chemiluminescence immunodetection system as described by the suppliers (Amersham International). The polyvinylidene difluoride membranes were exposed to X-ray film and the OD of the protein bands was measured using the Imaging Densitometer Model GS-670 (Bio-Rad) and the Molecular Analyst Software (Bio-Rad). The local background was substracted from each band. The protein content in cell extracts was determined using the Bio-Rad kit according to the manufacturers' instructions (DC protein assay, Bio-Rad Laboratories).

2.9. CDP reduction assay

The CDP reduction assay was conducted as previously described [21]. Briefly, 1×10^8 cells were pelleted, washed in PBS and resuspended in a hypotonic buffer containing 20 mM Tris, pH 7.6, 10 mM $MgCl_2$ and 2 mM DTT. Cells were thawed once and homogenized in a Dounce homogenizer with a tight fitting pestle and centrifuged for 30 min at 100,000 *g* at 4°. The proteins were precipitated by adding saturated ammonium sulfate and were then pelleted. The proteins were resuspended in 50 mM Tris–HCl, pH 7.5 and desalted with a Bio-Spin Chromatography Column (Bio-Rad). The reaction mixture contained 8 mM Hepes, pH 7.5, 6.4 mM $MgCl_2$, 3 mM ATP, 10 mM DTT, 0.01 mM $FeCl_2$, 20 mM KCl, 0.5 mM ^{3}H-CDP and 200 μg protein in a total volume of 100 μL. The reaction was incubated at 37° for 30 min and terminated by addition of 0.5 mL 1 M PCA. dCMP was added to the reaction prior to centrifugation, then the supernatant was boiled for 10 min to yield CMP and dCMP. Samples were then neutralized with KOH and dCMP was separated from CMP using a Dowex-50 column. The amount of the added dCMP remaining after separation was quantified by measuring the absorbance at 260, 280 and 310 nm. The amount of CDP reduction was calculated from the

radioactivity in the dCMP fraction, the amount of added dCMP being taken into account. CDP reduction was expressed in nM. Allosteric regulation of RR was studied by adding dATP (400 μM), CdATP (500 nm), didox (50 μM) or HU (500 μM) to the reaction.

2.10. Spectral karyotyping (SKY)

Slides with metaphase spreads were freshly prepared according to standard cytogenetic procedures prior to SKY hybridization. Slides were fixed in methanol:acetic acid (3:1) for 40 min, soaked in acetone for 10 min, dehydrated through an ethanol series and air-dried. The slides were pretreated with proteinase K for 3–7 min followed by denaturation at 72° in denaturing solution (70% formamide, 2× standard saline citrate (SSC) pH 7.0), dehydrated through an ice-cold ethanol series and air-dried. The 24 differentially labeled chromosome specific painting probes (SKY kit from Applied Spectral Imaging (ASI), and Cot-1 DNA (Invitrogen), were denatured and hybridized to denatured metaphase chromosomes from the leukemic cell lines according to the manufacturer's recommendation (ASI). After hybridization at 37° for 48 hr, slides were washed in washing solution containing 50% formamide and 2× SSC. The chromosomes were then counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vysis Inc.). Image acquisitions were performed using a SD200 Spectracube system (ASI) mounted on a Zeiss Axioskop microscope with a custom designed optical filter cube (SKY-1, Chroma Technology), that allows for the simultaneous excitation of all dyes and the measurement of their emission spectra. Spectrum-based classification of the raw spectral images was performed using Sky View 1.5 software (ASI). For each case, at least 10 metaphases were analyzed by SKY. Reversed DAPI banded gray scale images of the same chromosomes were captured separately and contrast-enhanced using Sky View 1.5.

2.11. Comparative genomic hybridization (CGH)

CGH was basically performed as previously described [22]. In brief, test DNA samples from leukemic cell lines were labeled with fluorescein isothiocyanate (FITC)-12-dUTP (Dupont) by nick translation. Normal reference DNA was labeled with Spectrum Red (Vysis Inc.). Test and normal DNA were mixed with unlabeled Cot-1-DNA (Invitrogen), denatured and applied onto slides with denatured metaphases from normal lymphocytes (Vysis Inc.). After hybridization at 37° for 48 hr, slides were washed in 0.4× SSC/0.3% Nonidet P-40 at 74° for 2 min, in 2× SSC/0.1% Nonidet P-40 at room temperature for 2 min and in sterile autoclaved water for 2 min. After air-drying, slides were counterstained with DAPI (Vysis Inc.).

Eight to 11 three color digital images (DAPI, FITC and Spectrum Red fluorescence) were captured from each hybridization using an Axioplan 2 (Carl Zeiss Jena GmbH) epifluorescence microscope equipped with a Sensys (Photometrics) cooled charge-coupled device (CCD) camera interfaced to an IPLab Spectrum 10 workstation (Signal Analytic Corp.), as previously described [23]. Between 12 and 20 ratio profiles (except for X and Y) were averaged for each chromosome to reduce noise. Green to red ratios greater than 1.2 were considered as gains, and ratios less than 0.8 were considered as losses. Heterochromatic regions in the centromeric and paracentromeric parts of the chromosomes, the short arm of the acrocentric chromosomes, the Y-chromosomes and all regions just next to the telomeres were not included in the evaluation.

2.12. Statistical analysis

The data are expressed as mean and standard deviations. Data was evaluated using the Student's *t*-test using StatView IV (Abacus Concepts Inc.). A probability value of less than 0.05 was considered statistically significant.

3. Results

3.1. Induction of resistance to CdA, CAFdA and Fara-A and resistance phenotypes

In order to study the mechanism of resistance to CdA, CAFdA and Fara-A, the T-cell leukemia cell line CCRF-CEM was treated with increasing concentrations of the respective drugs. The resistant cell lines were characterized regarding cell cycling and doubling time. The cells exhibited similar cell cycling behavior during the logarithmic growth phase, neither the percentage of cells in G1 and S phase nor the doubling times differing significantly compared to wild type cells (data not included). The resistance phenotypes for CEM/CdA, CEM/CAFdA and CEM/Fara-A are summarized in Table 1. The CdA and CAFdA resistant cell lines showed cross-resistance to other antimetabolites phosphorylated by dCK and dGK; Fara-A, Ara-C, dFdC, Ara-G, difluorodeoxyguanosine and deoxyadenosine. The Fara-A resistant cell lines were cross-resistant to CdA, Ara-C, dFdC and deoxyadenosine, but to a lower extent than the CdA and CAFdA resistant cells, although they preserved their sensitivity to CAFdA, Ara-G and difluorodeoxyguanosine. There was no cross-resistance for the resistant cell lines to tubercidin, which is a nucleoside transport inhibitor (Table 1) or to daunorubicin (data not shown).

3.2. Drug accumulation, phosphorylating and dephosphorylating enzymes

Cells were incubated with CdA, CAFdA, Ara-C and Fara-A for 2 hr and the accumulation of the corresponding triphosphates was analyzed by HPLC. The CdA and CAFdA resistant cell lines had nondetectable levels of

Table 1
Cytotoxicity data and resistance factors (Rf) of parental and CdA, CAFdA and Fara-A resistant CCRF-CEM cells

	CEM/wt (IC_{50}, μM[a])	CEM/CdA (IC_{50}, μM (Rf)[b])	CEM/CAFdA (IC_{50}, μM (Rf))	CEM/Fara-A (IC_{50}, μM (Rf))
CdA	0.14	>500[c] (>3700)	403 (2875)	18 (129)
Fara-A	6	>500 (>80)	>500 (>80)	175 (29)
CAFdA	0.020	>500 (>25000)	>500 (>25000)	0.026 (1)
Ara-C	0.04	152 (3451)	233 (5299)	1.4 (31)
dFdC	0.01	17 (1559)	29 (2601)	0.10 (9)
Ara-G	11	104 (10)	123 (11)	13 (1)
Difluorodeoxyguanosine	1.5	>60 (<39)	>60 (<39)	1.4 (1)
Deoxyadenosine[d]	1.6	9 (6)	11 (7)	19 (12)
Tubercidin	0.37	0.29 (1)	0.30 (1)	0.33 (1)

[a] The experiments were repeated at least twice and the data represents one experiment in triplicate. The intra-assay variation was less than 20%.
[b] Rf, resistance factor.
[c] Greater sign ('>') denotes the highest concentration used in the assay.
[d] For deoxyadenosine the cells were preincubated with 10 μM EHNA for 1 hr to inhibit adenosine deaminase.

Table 2
Cellular metabolites and metabolic enzymes in CCRF-CEM cells resistant to CdA, CAFdA and Fara-A and the wild type

	CEM/wt	CEM/CdA	CEM/CAFdA	CEM/Fara-A
Triphosphate formation (μM/hr) after incubation with				
1 μM CdA	17 ± 1	n.d.[a]	n.d.	20 ± 1
1 μM CAFdA	118 ± 7	n.d.	n.d.	136 ± 7
5 μM Ara-C	138 ± 11	n.d.	n.d.	101 ± 5
30 μM Fara-A	111 ± 5	n.d.	n.d.	61 ± 2
dCK (dCyd as substrate)	231 ± 8[b]	6 ± 2	5 ± 0.5	185 ± 65
dGK (Ara-G as substrate)	45 ± 6	56 ± 14	54 ± 5	65 ± 5
High K_m 5′-NT (IMP as substrate)	2242 ± 141	4438 ± 682	3734 ± 684	5018 ± 104

[a] n.d.: triphosphate levels are under detectable levels (less than 200 nM).
[b] Data are expressed as pmol per mg protein per minute.

triphosphates of all four analogues (Table 2). The Fara-A resistant cells displayed higher accumulation of triphosphates of CdA and CAFdA while the accumulation of Ara-C and Fara-A nucleotides was reduced to 73 and 55%, respectively (Table 2). We measured the activity of the phosphorylating and dephosphorylating enzymes in our cell lines using a radioactive substrate-based technique (Table 2). dCK activity was measured with dCyd as a substrate. In CdA and CAFdA resistant cell lines it was reduced to less than 5% of the dCK activity of wild type

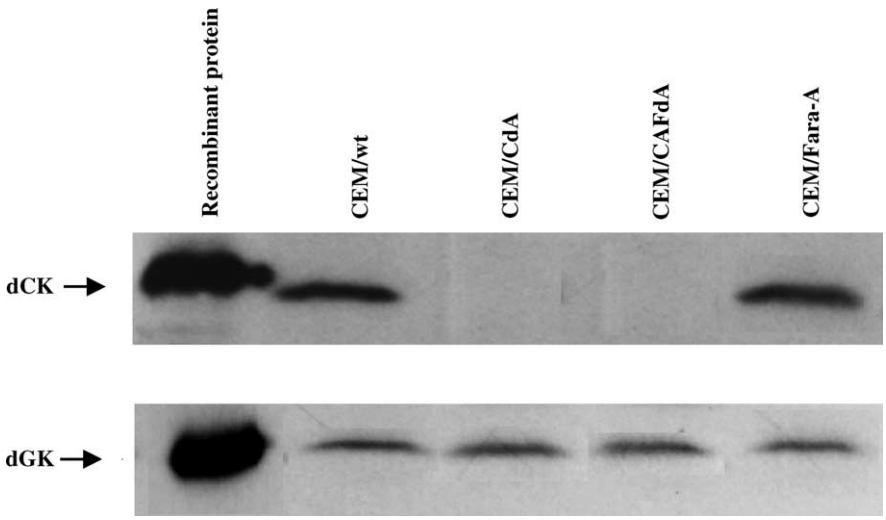

Fig. 2. SDS–PAGE and subsequent immunoblot analysis for dCK and dGK in resistant and parental cell lines. Cell extracts (40 μg) were loaded and separated by SDS–PAGE. Immunoblotting was performed using polyclonal antibodies against dCK and dGK as described in Section 2. Recombinant protein was loaded in the first lane. The dCK protein is represented by the 30 kDa band and the dGK protein by the 28 kDa band. The procedure was repeated at least three times using different extracts.

cells while the Fara-A resistant cells retained an average 80% of initial dCK activity. Using immunoblot analysis we were unable to detect any visible band for dCK protein in CdA and CAFdA resistant cell lines, although the Fara-A resistant cell line had equal amount of dCK protein as parental cells (Fig. 2). The residual dCK activity for CdA and CAFdA resistant cell lines may derive from TK2 activity, since TK2 also phosphorylates dCyd. dGK activity was measured using Ara-G as substrate. It was increased in all resistant cell lines (120–150% compared to parental cells). The dGK protein content did not practically differ in resistant and wild type cells (Fig. 2). We measured the activity of high K_m 5′-NT with IMP as substrate (Table 2). It was 2242 pmol/min/mg protein activity in extracts for CEM/wt cells and was increased 2-, 1.7- and 2.2-fold in the CEM/CdA, CEM/CAFdA and CEM/Fara-A resistant sublines, respectively. The mRNA levels of the phosphorylating and dephosphorylating enzymes were measured by real-time quantitative PCR. We determined that in CdA and CAFdA resistant cells the mRNA levels of dCK were substantially reduced compared to the parental cell line, while dGK and 5′-NT mRNA levels were slightly diminished (CEM/CdA) or unchanged (CEM/CAFdA). For Fara-A resistant cells the mRNA levels for dCK, 5′-NT and dGK were equal to those of wild type cells (data not included). The coding region of dCK was sequenced using the pCR®II-TOPO® vector. No mutations were detected in any of the resistant cell lines (data not included).

3.3. Ribonucleotide reductase and reversal of resistance with RR inhibitors

Altered levels of RR activity have been reported to be a possible cause of resistance to dFdC [24]. As evident in Fig. 3A, we did not observe any changes in the protein levels of the large subunit R1. The level of small subunit R2 in CEM/CdA cells was also equal to that of CEM/wt, while the expression of R2 protein was elevated in the Fara-A resistant cells and was substantially reduced in the CAFdA resistant cell line. The total intensity of the R2 bands was measured and was 91, 15 and 143% for the CdA, CAFdA and Fara-A resistant cell lines, respectively, as compared to the wild type. Using the CDP reduction assay we detected a 40% increase in the RR activity in Fara-A resistant cells compared to that of the wild type (Fig. 3B). The RR activity in CdA and CAFdA resistant cells was reduced to 84 and 53% of the activity in CEM/wt, respectively (Fig. 3B). As the alteration in allosteric regulation of RR may also be a possible reason for development of resistance to nucleoside analogues, we investigated it by using the RR inhibitors dATP, CdATP, didox or HU. No difference in the effectivity of RR inhibition was found between the resistant and wild type cells (data not included).

A new protein, p53R2, which is directly induced by p53 has been discovered that together with the R1 subunit can reduce ribonucleotides to their corresponding deoxyribonucleotides *in vitro* [25,26]. To examine a possible role of p53R2 in substituting of R2 in the CdA and CAFdA resistant cells, we measured p53R2 protein levels by immunoblotting. As displayed in Fig. 3A, for CAFdA resistant cells there was no detectable band for the full-size p53R2 protein, the CdA and Fara-A resistant cells having 89 and 120% intensity of the p53R2, respectively, compared to the wild type. For the CAFdA resistant cells a protein with lower molecular weight was detected with the

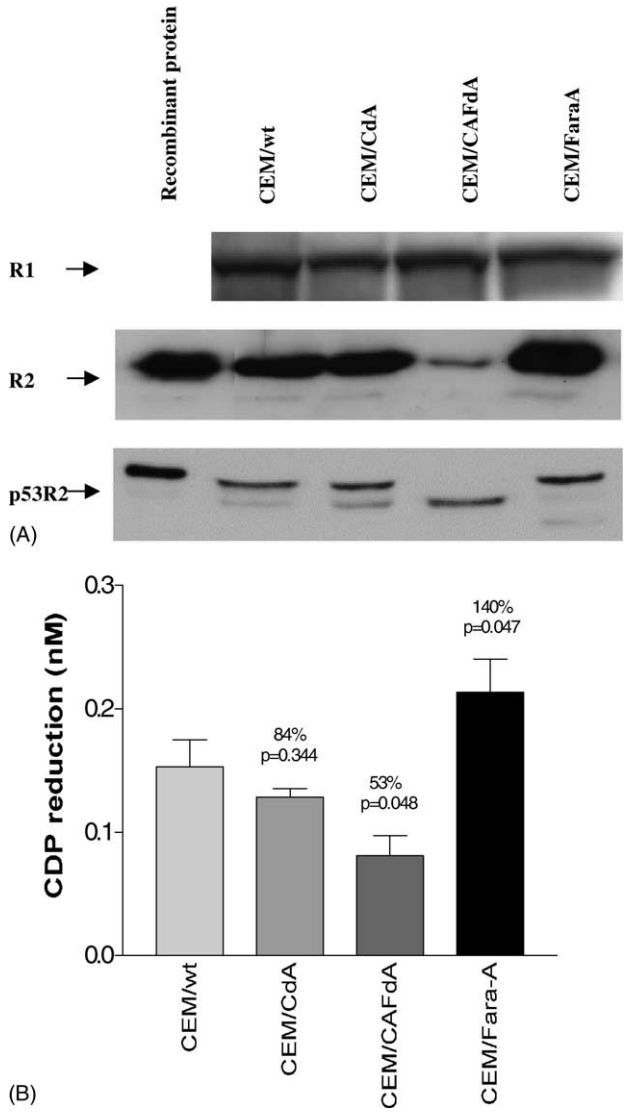

Fig. 3. (A) SDS–PAGE and Western blot analysis of the two subunits of ribonucleotide reductase; R1 and R2 and the p53 induced subunit p53R2 for the CEM/wt and the derived CdA, CAFdA and Fara-A resistant cell lines. The crude RR cell extracts (30 μg for R1, 10 μg for R2 and 15 μg for p53R2) were loaded into each lane. For R2 and p53R2 a recombinant protein was loaded in the first lane. The R1 subunit is represented by the 85 kDa band, the R2 subunit by the 44 kDa band and the p53R2 subunit by the 40 kDa band. The procedure was repeated at least three times using different extracts. (B) Ribonucleotide reductase activity, measured as the formation of dCDP in crude RR enzyme extracts from CdA, CAFdA, Fara-A resistant CEM cell lines and the wild type. The percentage of RR activity compared to the wild type cell line and the statistical significance (*P*-value) is shown. The CDP reduction assay was performed as described in Section 2. The experiment was repeated three times.

Table 3
Chromosomal aberrations detected by CGH in CCRF-CEM wild type and its resistant sublines

CEM/wt	+1p32p36,-9p21p24,+20
CEM/CdA	−X,+1p32p36,-2q21q37,-4,-9p21p24,+20
CEM/CAFdA	−Xq21q28,+1p32p36,-9p21p24
CEM/Fara-A	−X,+1p32p36,+2p11p25,-4,-6,-9p21p24,+17,+20

Chromosomal abberations detected by SKY in CCRF-CEM wild type and its resistant sublines

CEM/wt	92-95⟨n⟩XX,dup(1)(p32p36),t(8;9)(p11.2;p24)x2,del(9)(p21),+11,del(11)(q23)x2,+20,+20[cp10]
CEM/CdA	83-88⟨n⟩XX,-X,-X,dup(1)(p32p36),der(2)t(2;13)(q21;q11),-4,-9,del(9)(p21),der(10)t(10;20)(p11.2;q11.2),del(11)(q12),-13,-21[cp10]
CEM/CAFdA	83-89⟨n⟩XX,del(X)(q21),der(X)t(X;10)(q22;q22),dup(1)(p32p36),del(1)(q25),del(4)(q12),del(6)(q22),t(8;15)(q21.2;p11.1),-9,del(9)(p21),del(10)(q22),del(11)(q23),-13,-15,-16,-18[cp10]
CEM/Fara-A	86-89⟨n⟩XXX,-X,dup(1)(p32p36),dic(2;20)(p10;p10)x2,-4,-6,-9,del(9)(p21),-15,-16[cp10]

p53R2 antibody, this protein having the same intensity in CEM/CAFdA as the full-length p53R2 in the wild type. The intensity of the short band detected by p53R2 antibody was low in all cell lines (<25% of the full-length p53R2 in the wild type) except for CEM/CAFdA. The sum of the intensity of the R2, p53R2 and short p53R2 bands for the different cell lines mirrored the RR activity.

Since one rationale to overcome cellular resistance to the nucleoside analogues studied here may be the use of the RR inhibitor didox, we combined didox with Fara-A and measured the IC_{50} values. The CEM/Fara-A cells treated with Fara-A in combination with didox (50 μM), yielded more than 20-fold reduced IC_{50} value ($IC_{50} = 7.7$) as compared to the CEM/Fara-A cells treated with Fara-A alone (Fig. 4), nearly the same levels as for the wild type treated with Fara-A ($IC_{50} = 6.0$).

3.4. Cytogenetic results

As analyzed by SKY, the wild type cell line and the resistant sublines possessed a near tetraploid karyotype, sharing several numerical and structural aberrations (Table 3). However, net losses of Xq21-qter, 4q12-qter as well as a loss of the entire chromosome 9 were only observed in the three resistant cell lines. The CGH results contained a gain of the distal part of 1p and a loss of the distal part of 9p in all four cell lines analyzed, as well as a loss of the distal part of Xq in all three resistant cell lines. Notably, the resistant cell lines showed a more complex picture of gains and losses compared to the wild type.

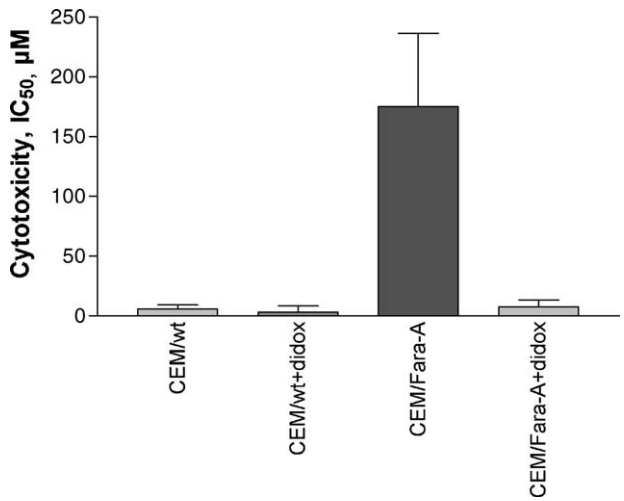

Fig. 4. MTT analysis of the Fara-A resistant CEM cell line and the wild type. The IC_{50} value for the Fara-A resistant cells when incubating cells with Fara-A in combination with the ribonucleotide reductase inhibitor didox is reduced more than 20-fold, as compared to incubation with Fara-A alone. The experiments were repeated three times in triplicate and representative data from one experiment is depicted.

4. Discussion

A major problem in the treatment of hematological malignancies is the development of resistance to chemotherapy. It is therefore important to delineate the mechanisms of resistance to CdA and Fara-A and to compare it with that developed to CAFdA. CdA and Fara-A are active against both replicating and nonreplicating cells. Several studies suggest the absence of cross-resistance between these two analogues [27,28]. CAFdA, a newly synthesized analogue of CdA and Fara-A, has major activity against various leukemic cells [29–31] with a potential to substitute for CdA and Fara-A as an effective drug. DCK activates several important nucleoside analogues which have been shown to vary extensively in leukemia patients and which are not always indicative of *in vivo* responses [32,33].

In this study, we detected a deficiency of dCK at the level of enzymatic activity, protein level and mRNA expression for the CdA and CAFdA resistant cells, and these cell lines did not form detectable levels of CdATP, CAFdATP, Ara-CTP or Fara-ATP. An earlier study showed that resistance to Ara-C in human T-lymphoblasts was mediated by mutations within the dCK gene [34]. We did not detect any mutations in the coding region of dCK or spliced fragments of dCK in any of the resistant cell lines in this study. It has previously been reported that Fara-A resistant cells have a low accumulation of Fara-A nucleotides, and to some extent a decreased activity of the activating enzyme dCK [7,35]. However, the mechanism of resistance to Fara-A was not further studied and is therefore not yet clarified. Our results suggest that the mechanism of

resistance to Fara-A seems to be different from that of resistance to CdA and CAFdA, since dCK was slightly lower while the RR activity were substantially higher in these cells. dCK is the main phosphorylating enzyme for these three nucleoside analogues; however, CdA and CAFdA are much better substrates for dCK than Fara-A, as previously indicated by K_m and V_{max} values for these compounds [36]. Thus, other aberrations must contribute to resistance against Fara-A.

The activity of RR was higher in these cells and the protein level of the small subunit R2 was increased. Earlier studies have shown that introduction of HU resistance confers overexpression of the R2 mRNA and protein levels [37]. A KB cell line resistant to dFdC had slightly lower dCK activity but a clear-cut increased of RR activity, due to high expression of the small subunit R2 [24]. It has previously been shown that in the human erythroleukemic cell line K562, the RR activity was slightly increased in cells resistant to CdA and Fara-A [38]. The Fara-A resistant cell lines in our study displayed a similar pattern of resistance, i.e. an up-regulation of RR activity and increased levels of the R2 subunit. The overall regulation of RR may be disrupted, as previously reported [39], but we found no evidence for this, since dATP, CdATP, didox, and hydroxyurea inhibited the enzyme activity in the different cell lines in a similar way as the wild type enzyme. Our results suggest that a contributing factor in Fara-A resistance is increased RR activity due to higher level of the R2 protein. One rationale to circumvent the resistance may be to combine nucleoside analogues with RR inhibitors. When Fara-A was combined with didox the cytotoxic effect of Fara-A was enhanced in the Fara-A resistant cells; therefore this may be an approach to overcome resistance to Fara-A. HU and didox have also been combined with Ara-C, leading to increased cytotoxicity [11,40]. The combination of RR inhibitors with Fara-A remains to be investigated *in vivo*. Since the sensitivity to Fara-A when combined with didox was highly increased, it is likely that this is the most important resistance mechanism for the Fara-A resistant cells.

It was recently shown that high levels of mRNA of high K_m 5′-NT independently identify a poor prognosis group of patients with acute myeloid leukemia [41]. We determined a 2-fold increase in the high K_m 5′-NT activity for the resistant cell lines. The mRNA expression of 5′-NT was not increased for the resistant cells. It has recently been demonstrated that expression of recombinant 5′-NT (cN-I) in leukemic cell lines conferred resistance to CdA [42]. Little information regarding the relative importance of different 5′-NTs in the dephosphorylation of nucleoside analogues and remains to be investigated.

Surprisingly, the CdA and CAFdA resistant cell line had lower RR activity and undetectable R2 protein band in CAFdA cells compared to the wild type. Since the CdA and CAFdA resistant cells had similar doubling time and percentage of cells in S phase as the wild type and showed a deficiency in dCK activity, the question arises of how the dNTPs needed for dividing cells are produced. Recently, a new p53-induced R2 protein was discovered, p53R2 [25] that forms an active complex *in vitro* with subunit R1 [26]. We hypothesized that the p53R2 protein could substitute for R2 in the CdA and CAFdA resistant cells but this appears not to be the case since p53R2 was not up-regulated in these cells. However, there was a smaller protein band detected by the p53R2 antibody that was several-fold increased in the CdA and CAFdA resistant cells. It is not known whether this band is a splice variant of p53R2 or if it has any activity. One hypothesis could be that this splice variant, together with R1, may supply the cell with the dNTPs. An earlier study by Chitambar *et al.* showed that CEM cells resistant to gallium had decreased CDP reductase activity but no decrease in the rate of proliferation [43]. These results concord with our results for the CAFdA and CdA resistant cells, the CDP reductase activity being decreased but the cells still maintaining the same proliferation rate as CEM/wt cells.

DCK was mapped to chromosome 4, dGK, 5′-NT, thymidine kinase 1 and thymidine kinase 2 being located on chromosomes 2, 6, 17 and 16, respectively. The two subunits of RR, R1 and R2 are located on chromosomes 11 and 2. In order to investigate the effect of nucleoside analogues at the chromosomal level, we performed a molecular cytogenetic analysis using SKY and CGH. In general, the three resistant cell lines shared a more complex picture of rearranged chromosomes compared to wild type. The CAFdA resistant cell line exhibited the highest number of structurally rearranged chromosomes, indicating a high potential of CAFdA to rearrange the genome. It is interesting to note that the resistant sublines shared a net loss of the distal part of the long arm of chromosome 4, encompassing the region for the *DCK* gene at 4q13.3-q21.1. According to the cytogenetic data three of the four chromosome 4 remain intact and it is therefore unlikely that a loss of the dCK region should result in a down-regulation of the *DCK* gene in these tetraploid cells. Other genetic mechanisms involving the down-regulation of mRNA expression of dCK are more likely to be involved in the dCK deficiency, as detected for the CdA and CAFdA resistant cells. The mechanism of dCK deficiency is largely unknown. One possible mechanism could be DNA methylation, which regulates gene expression in mammalian cells [44]. However, we have previously shown that CCRF-CEM cells resistant to CdA and CAFdA contained no methylated cytosine in the dCK gene promotor [45].

Other contributing factors to resistance could be decreased influx or increased efflux of the drug. Since dCK is not altered so much in the Fara-A resistant cells the influx of the drug may be disrupted. However, the absence of cross-resistance to tubercidin, which is transported by the same transporter as Fara-A, and the high level of accumulation of the triphosphates of nucleoside analogues implicates that the transport is not affected. The multidrug

resistance can be caused by overexpression of P-gp (Mdr1) or MRP in mammalian cells. The resistant cell lines included in this study did not display cross-resistance to antracyclines. Nevertheless, it was recently speculated that MRP5 might play a role in resistance to nucleoside analogues, based on resistance to nucleoside analogues in MRP5-transfected cells [46]. We therefore suspected that the up-regulation of multidrug resistance proteins and efflux of the drug could be an additional contribution for resistance to Fara-A. We investigated P-gp and MRP1–6 but detected no up-regulation of these proteins, thus ruling them out as a mechanism for resistance.

The cytotoxicity of nucleoside analogues ultimately ends with apoptosis. Two different pathways for the induction of apoptosis have been reported, the death receptor pathway, such as *via* Fas/Fas ligand, or the mitochondria-dependent mediated activation of apoptosis. Cytochrome *c* is released from the mitochondria and binds together with dATP/CdATP that function as a co-factor to the apoptosis protein activating factor-1 (APAF-1). This complex, the apoptosome, induces apoptosis *via* caspase-9 activation [47]. The potency of different co-factors to induce apoptosis was investigated, Fara-A being determined to be a more potent co-factor than CdA [48]. In a previous study we found that CEM cells resistant to CdA and Fara-A did not release cytochrome *c* from the mitochondria upon treatment with CdA and that resistant cells did not show an increase in intracellular Ca^{2+} release [49]. The differences in potency as co-factors between these three nucleoside analogues can also contribute to the differences in effect and it is clearly documented that CdA and Fara-A are not equally effective in various leukemias. This may also contribute to a different pattern of resistance, as clearly demonstrated in this study.

In conclusion, it was shown that the nucleoside analogues CdA, CAFdA and Fara-A have different patterns of resistance. The mechanism for resistance to Fara-A seems mainly due to RR activity. The observation that Fara-A resistant cells were sensitive to CAFdA and that this was reversed by a combination of didox with Fara-A is a novel finding and may be important in the treatment of patients with refractory hematological malignancies. In contrast, the most important reason for resistance to CdA and CAFdA is the drastically decreased level of the main nucleoside phosphorylating enzyme, dCK. Our results also clearly indicate that the level of the truncated form of p53R2 varied in these cell lines and may to some extent substitute for R2 in the production of dNTPs in the resistant cells.

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