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Decreased Resistance to Gemcitabine (2′,2′-Difluorodeoxycitidine) of Cytosine Arabinoside-Resistant Myeloblastic Murine and Rat Leukemia Cell Lines: Role of Altered Activity and Substrate Specificity of Deoxycytidine Kinase

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ABSTRACT. We determined the potential activity of 2′,2′-difluorodeoxycytidine (gemcitabine, dFdC) in 1-β-D-arabinofuranosylcytidine (ara-C)-sensitive and-resistant leukemia cell lines. Both drugs are phosphorylated by deoxycytidine kinase (dCK); the triphosphates, dFdCTP and ara-CTP, respectively, are incorporated into DNA. In the murine leukemia cell line L1210, induction of resistance to ara-C resulted in the 2200-fold resistant subline L4A6. The Brown Norway rat myelocytic leukemia ara-C-sensitive cell line (BCLO) was >300-fold more sensitive to ara-C than its variant Bara-C. In L1210 cells, gemcitabine was 8-fold more active than ara-C; in L4A6, BCLO, and Bara-C cells, gemcitabine was 16-, 28-, and more than 3-fold more active than ara-C, respectively. A partial explanation for these differences may be the higher dCK activity in the parental cell lines L1210 and BCLO with gemcitabine compared to ara-C as a substrate. DCK activity was not or hardly detectable in the resistant L4A6 and Bara-C cell. In the rat leukemia cell lines, deoxycytidine (dCyd) phosphorylation activity showed an aberrant pattern, since the activity with dCyd was 1.5-fold higher in the Bara-C cell line compared with BCLO, possibly due to thymidine kinase 2. The wild-type L1210 cells accumulated at least 3-fold more ara-CTP and dFdCTP than the rat leukemia cell line BCLO. The ara-C-resistant variants L4A6 and Bara-C did not accumulate dFdCTP or ara-CTP. In conclusion, gemcitabine was more active than ara-C in all leukemia cell lines tested. The sensitivity of the wild-type cell lines correlates with the accumulation of dFdCTP and ara-CTP, but is independent of dCK. However, both resistant variants had decreased dCK activities, but were relatively more sensitive to dFdC than to ara-C. BIOCHEM PHARMACOL **57**;4:397–406, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. gemcitabine; ara-C; drug resistance; cytogenetic alterations; drug accumulation; deoxycytidine kinase activity; thymidine kinase activity

dFdC** is an antineoplastic agent with activity against several experimental solid tumors, including non-small cell lung cancer and ovarian carcinoma and in the clinic [1–5]. However, clinical studies in leukemia were discontinued. After entering the cell, the drug has to be phosphorylated by dCK to dFdCMP and subsequently to dFdCTP, which can be incorporated both into DNA and RNA [6–8]. Gemcitabine can be inactivated by the action of dCDA to 2′,2′-difluorodeoxyuridine [6]. Like gemcitabine, ara-C is a deoxycytidine analogue and is the major drug in the treatment of acute leukemia. In addition, ara-C requires phosphorylation by dCK in order to be active, and can be inactivated by deamination by dCDA to 1-β-D-arabinofuranosyluridine. The mitochondrial enzyme TK2 can also phosphorylate the natural nucleosides TdR, dCyd, and UdR. This is in contrast to the cell-cycle dependent cytosolic enzyme TK1, which phosphorylates only TdR and

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** *Abbreviations:* ara-C, 1-β-D-arabinofuranosylcytidine; Bara-C, ara-C-resistant BNML; BCLO, ara-C-sensitive BNML; BNML, Brown Norway rat myelocytic leukemia model; dCDA, deoxycytidine deaminase; dCK, deoxycytidine kinase; dCyd, deoxycytidine, dFdC, 2′,2′-difluorodeoxycytidine, gemcitabine; FBS, fetal bovine serum; TD, tumor doubling time; TdR, thymidine; TK1, thymidine kinase 1; TK2, thymidine kinase 2; and UdR, deoxyuridine.

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UdR but not dCyd [9]. Neither of the two enzymes has been shown to phosphorylate ara-C. Ara-C in its active form, 1-β-D-arabinofuranosylcytosine triphosphate, is a potent inhibitor of DNA synthesis by inhibition of DNA polymerase [10]. Although the treatment of acute myelogenous leukemia with ara-C is relatively successful, the eventual formation and/or selection of resistant tumor cells allows uncontroled regrowth of the tumor resulting in failure to cure [11–13].

Several possible mechanisms of ara-C resistance have been described previously, and it has not been completely clarified whether all these mechanisms would lead to resistance to dFdC. Decreased transport through the cell membrane has been related to ara-C resistance. At low concentrations, ara-C is taken up into the cell by facilitated diffusion, but at high concentrations ara-C enters the cell by diffusion, thus bypassing this form of resistance [14, 15]. Other mechanisms of ara-C resistance include dCK deficiency, decrease in the half-life of the active metabolite ara-CTP, and an increase in intracellular dCTP [16–18]. Since dCTP is an inhibitor of dCK and competes with ara-CTP for DNA polymerase, resulting in a decrease of ara-C incorporation into DNA, an increase of dCTP pools will decrease ara-C sensitivity [18, 19].

Myeloblastic leukemia cell lines, including those of murine and rat origin, are widely used cell systems for the testing of antitumor drugs. L1210 leukemia cells are highly sensitive to ara-C but may develop ara-C resistance during treatment [11]. This feature, which resembles the clinical response of leukemic cells, makes the model eminently suitable for investigations on the mechanisms by which sensitive cell populations change into drug-resistant cells following treatment. For this purpose, we developed resistant murine L1210 cell lines.

Brown Norway (BN) rat myelocytic leukemia was chemically induced in a female BN rat by means of 9,10-dimethyl-1,2-benzanthracene. This BN myelocytic leukemia model (BNML-O) is considered as an excellent model for human myeloid leukemia [20]. The ara-C-resistant variant of BNML-O (BNMLO-Ara-C) was developed by *in vivo* suboptimal ara-C treatment and repeated transplantation [21]. From both leukemia models, *in vitro* growing cell lines were derived; the ara-C-sensitive BNML-CL/O (BCLO) [22] and the ara-C-resistant BNML-CL/Ara-C (Bara-C). The resistance to ara-C is the result of an aberrant dCK activity [23].

Here, we describe the development of ara-C resistance in the murine leukemia cell line L1210, and the characterization of this ara-C-resistant variant L4A6. In order to determine whether ara-C-resistant murine and rat myeloblastic leukemia cell lines would be similarly resistant to gemcitabine, we tested L1210 cells and their ara-C-resistant variant L4A6, BCLO, and its ara-C-resistant variant Bara-C for sensitivity to gemcitabine. Analysis of the mechanisms of resistance indicated differences in the accumulation of dFdCTP and ara-CTP and abnormal substrate specificity of dCK in the rat leukemia cell lines.

MATERIALS AND METHODS

Chemicals

Gemcitabine (dFdC) was kindly supplied by Eli Lilly and Co. Ara-C was obtained from Upjohn. ^{3}H-dFdC (21 Ci/mmol) was a kind gift from Eli Lilly and Co. and produced by the Amersham Radiochemical Center, from which ^{3}H-ara-C (28.7 Ci/mmol) was also obtained; ^{3}H-dCyd (21.9 Ci/mmol) was from Moravek and ^{14}C-TdR (62.8 mCi/mmol) from NEN. RPMI 1640 was purchased from Flow Laboratories. Hoechst 33258 was obtained from Hoechst. All other chemicals were of analytical grade and commercially available.

Cell Culture

Murine leukemia cells derived from L1210 used as its variant L5 were routinely cultured in suspension in RPMI 1640 medium supplemented with 10% FBS, 60 μM 2-β-mercaptoethanol and 250 ng/mL gentamicin, as described previously [24]. BCLO cells were cultured in RPMI 1640 medium, as described [22], supplemented with 3.2 mM L-glutamine, 10% FBS, and 250 ng/mL gentamicin; Bara-C was cultured in the same medium as BCLO, but instead of 10% FBS, supplemented with 5% FBS and 5% rat serum. BCLO and Bara-C were generously provided by Dr L. Colly [then at the Dept. of Hematology, University Hospital Leiden, the Netherlands]. The ara-C-resistant cells were cultured in the presence or absence of 1 μM ara-C as indicated. All populations were cultured at 37° in a humidified atmosphere of 95% air and 5% CO_2 and regularly screened for mycoplasma contamination by using a rapid detection system with a ^{3}H-labeled DNA probe [Gen-Probe] and were found to be negative.

Induction of Resistance to Ara-C in L1210

Resistance to ara-C was induced by a stepwise increase in the drug concentration in the culture medium. The drug concentration was increased to the next higher value when no detectable inhibition of proliferation was observed. The ara-C concentrations used were 0.001, 0.05, 0.25, 0.50, and 1.00 μM in that order. Cells were considered to be ara-C-resistant when proliferation was undisturbed in the presence of 1 μM ara-C. This concentration was chosen because it can be reached in blood plasma of patients during treatment with intermediate dose ara-C [25]. Cells, however, also became resistant to ara-C levels (>10 μM) which are usually reached under high-dose ara-C [25].

Flowcytometry

Cells were pretreated with Triton X100 and RNAse and stained with propidium iodide. Analysis was performed using an Ortho System 30 Cytofluorograph (Ortho). The fluorescent DNA stain was excited using the 488 nm line of an argon ion laser.

Chromosome Analysis

Chromosome preparations of the L1210 variants were done after 30-min incubation with 10^{-8} g/mL vinblastine to arrest cells in mitosis. The cells were incubated in 0.075 M KCl for 7 min and fixed in 3:1 methanol:acetic acid fixative. Chromosomes were G-banded with the trypsin-Giemsa method modified according to Dofuku [26]. Simple Giemsa staining was performed to establish the presence of double minutes in mitotic cells.

Tumorigenicity

Female DBA/2 mice, 3 months old, were used as host to determine the tumorigenic potential of the L1210 and L4A6 cells. Three mice were used for each cell line. After an i.p. injection of 10^6 cells/0.2 mL PBS, the survival time, which is a measure of the tumorigenicity of the inoculated cells, was determined. The survival time varied between 9 and 10 days, while mice became sick at day 8 or 9 from either sensitive or resistant tumors. To establish the cause of death, the ascites of one mouse from each group was brought in culture and cytogenitically investigated. In all cases, the ascites cells could be identified as the inoculated leukemic cells.

Chemosensitivity Testing

Sensitivity to drugs was defined by the concentration of the drug causing a growth inhibition of 50% (IC_{50}) after 48-hr drug exposure of the cells. The cells were plated in 24-well plates in different densities, depending on their TDs. The optimal plating number was the highest number of cells possible to enable log linear growth for 48 hr (L1210, 100,000 cells/well; L4A6, 200,000 cells/well; BCLO, 300,000 cells/well; Bara-C, 400,000 cells/well) in a volume of 1 mL/well. The first column contained cells not exposed to drugs, while the cells in the second through the sixth column were exposed to increasing drug concentrations. Both gemcitabine and ara-C were added in a volume of 10 μL resulting in final concentrations of $1.10^{-9} - 1.10^{-4}$ M. After 48 hr, growth rate was determined by cell counting using a Sysmex Microcell counter; 0.5 mL of cell-containing medium was added to 9.5 mL of a 0.8% NaCl solution and counted. Growth inhibition curves were made relative to control of every assay. Two control values were always included; the number of cells plated at the day of drug administration (set at 0%) and the number of cells after 48 hr not exposed to drugs (set at 100%). The points were connected by straight lines, and the IC_{50} values were determined from the interpolated graph. The TD was determined from the logarithmic part of the slope.

Intracellular Ara-C Accumulation

Ara-C uptake through the cell membrane and subsequent intracellular accumulation was tested by the uptake of tritium-labeled ara-C during culture. 5-^{3}H-Ara-C (2μCi/mL) was mixed with cold ara-C and added to the culture medium resulting in a final concentration of 1 μM. After a 2-hr incubation of a logarithmically growing population with 5-^{3}H-ara-C at 37°, the cells were washed 3 times, lysed, and measured for the presence of tritium by scintillation counting according to routine methods. The ara-C accumulation was expressed as ^{3}H-counts per cell number.

dCK Enzyme Activities Measured with Different Substrates

For determination of dCK activities in cell lines, extracts (10,000 *g* supernatants) were used, prepared with cold dCK buffer (0.3 M Tris–Hcl, pH 8.0, 50 μM β-mercaptoethanol) essentially as described [27]. One part of the undiluted cell extract was taken for measurement of the protein content with the Bio-Rad Bradford protein assay [28]. The substrate mixture was prepared by mixing 2 vol. of Mg-ATP (50 mM ATP in 25 mM $MgCl_2$, pH 7.4), one vol. of dCK buffer and 2 vol. of tritiated substrate solution (for the final 230 μM dCyd, dFdC and ara-C concentration, the final specific activity was 0.04 Ci/mmol). To 25 μL of the substrate mixture, 25 μL supernatant ($0.2–4.0 \cdot 10^5$ cells) or 10 μL 50 mM TdR and 15 μL supernatant was added. TdR was added to inhibit TK2-mediated phosphorylation of dCyd [9]. The reaction mixtures were incubated at 37° for 30 min and terminated by heating at 95° for 3 min and subsequent addition of 10 μL 5 mM unlabeled dCyd, dFdC, or ara-C. The substrate (dCyd, dFdC, ara-C) was separated from the phosphorylated product (dCMP, dFdCMP, ara-CMP) by thin layer chromatography on polyethylene imine cellulose layers, with distilled water as eluent. The spots could be visualized, marked, and cut out. Radioactivity was estimated in a liquid scintillation counter after addition of 9 mL Optima Gold (Packard Instruments Co.). Enzyme activities were expressed as nmol product formed per hour per 10^6 cells (nmol/hr/10^6 cells). The detection limit using the above-mentioned conditions was about 0.04 nmol product formed/hr/10^6 cells.

dCDA Enzyme Activities Measured with Different Substrates

The activity of dCDA was determined as described earlier [27]. Briefly, 10,000 *g* supernatants were prepared and enzyme activity was determined at 37° in $1–5 \cdot 10^6$ cells with 500 μM dCyd, dFdC, or ara-C as a substrate. After a 15- or 25-min incubation, the reaction was terminated by precipitating the proteins by addition of 40% w/v trichloroacetic acid. The supernatant was neutralized with trioctylamine/1,1,2-trichloro-trifluoroethane (v/v:4/1). The upper aqueous layer containing the substrate dCyd, dFdC or ara-C and their respective products UdR, 2′,2′-difluorodeoxyuridine or 1-β-D-arabinofuranosyluridine was analyzed using reversed phase HPLC [27].

Thymidine Kinase Activities

TK1 and TK2 activities were determined as described for dCK. Supernatants (10,000 *g*), prepared with cold TK buffer (50 mM Tris–HCl, 1 mM EDTA, pH 7.4), were used to determine the enzyme activity and protein content with the Bio-Rad Bradford protein assay [28]. To 25 μL substrate solution (21.9 μM TdR, final specific activity 1.8 Ci/mmol; 20 mM ATP; 10 mM $MgCl_2$; 10 mM Tris; 200 μM EDTA), 25 μL enzyme-containing supernatant or 10 μL of 50 mM dCTP (an excess inhibiting TK2 [29] but not TK1) and 15 μL enzyme were added, and incubated at 37°. The reaction was stopped by heating the mixture at 95° for 3 min, followed by the addition of 10 μL of 5 mM TdR, 5 mM thymine. Substrate (TdR) and product (TMP) were separated by thin layer chromatography on polyetheleneimine cellulose layers, with distilled water as eluent. Radioactivity was estimated as described for the dCK enzyme assay.

dFdCTP and Ara-CTP Accumulation

Cells were plated in 6-well plates [Costar Corp.] at a density of $5 \cdot 10^6$ cells/well/2 mL medium. dFdC or ara-C was added to a final concentration of 10 μM. The cells were harvested and washed after 2 hr of incubation. Alternatively, cells were cultured in 25 cm^2 flasks for 4 hr. Thereafter, the nucleotides were extracted as previously described [30, 31]. Briefly, cell pellets were resuspended in 150 μL cold PBS (pH 7.4), and subsequently 50 μL ice-cold 40% trichloroacetic acid (A) (w/v) was added. The suspension was kept on ice for 20 min. Proteins were spun down (5 min, 12,000 *g*, 4°) and the supernatant was neutralized with 400 μL tri-octylamine/1,1,2-tri-chloro-trifluoroethane (v/v:1/4). Finally, dFdCTP was analyzed on HPLC using a Partisphere SAX [Whatman] column with a linear gradient between 5 mM $NH_4H_2PO_4$ (pH 2.8; buffer A) and 0.5 M $NH_4H_2PO_4$/0.25 M KCl (pH 3.0; buffer B; 35–100% B over 30 min) at a flow rate of 1.5 mL/min [30]. For ara-CTP determination, isocratical anion exchange HPLC was used (0.25 M KH_2PO_4 containing 0.5 M KCl (pH 4.5) at a flow of 1.5 mL/min). The retention time of ara-CTP was: 5.4 min. Nucleotides were detected at 254 and 280 nm [31].

RESULTS

Induction of Resistance to Ara-C and Characterization of the Resistant Cell Line

Exposure of L1210 leukemia wild-type cells to increasing ara-C concentrations yielded different resistant strains at the final 1 μM ara-C concentration. These strains differed in resistance, DNA content, chromosome number, and chromosome stability (Table 1). L4A6, in which resistance was achieved after 120 days of ara-C exposure, was chosen for gemcitabine sensitivity screening, since this cell line appeared to be stable and diploid like its parental cell line L1210. In other resistant subclones, differences in ploidy and severe chromosome deletions were found. For example, L5-T2 appeared to be pseudo tetraploid with 70–80 chromosomes and L5-ara6′ hyperdiploid having 41 chromosomes. After induction of ara-C resistance, the L1210 cells changed from a slow-growing into a stable fast-growing resistant population L4A6, whose population TD was only increased at 2 hr compared to its parental cell line (Table 1). L1210 and L4A6 are fast-growing cell lines in comparison with BCLO and Bara-C, with TDs of 28.0 ± 5.8 hr and 28.1 ± 3.5 hr, respectively.

TABLE 1. Characteristics of the L1210 and L4A6 murine leukemia cell lines

	L1210	L4A6
TD (hr)	15.0 ± 1.3	17.2 ± 00.9
DNA content	Diploid	Pseudo diploid
Chrom. number	38–40	39
Chrom. stability	Intracell rearrangements	Nondisjunction
Tumorigenicity	++	++

DNA profiles of the L1210 and L4A6 cells in logarithmic phase indicated identical DNA contents (Fig. 1), but a shift in the distribution of the cells over the cell cycle was observed; 27% of the L4A6 cells were in the G2/M phase, compared to 17% of the L1210 cells. However, no difference in DNA content and distribution between the ara-C-resistant hyperdiploid L5-ara6′ cells and its parental L1210 cells was found, but the DNA content in the pseudo tetraploid L5-T2 cells was more than 2-fold that of the L1210 cells (Fig. 1). L4A6 remained fairly stable with respect to their DNA content for more than 6 months of culture with or without ara-C. Cytogenetic analysis of the L1210-sensitive parental and the ara-C-resistant variant L4A6 revealed a variable number of chromosomes in the parental cell line L1210, whereas L4A6 appeared to be stable with regard to their chromosome number of 39. This was the major reason for choosing L4A6 for further experiments. The karyotype of L1210 cells involves a remarkable number of variable aberrations such as gaps, deletions, translocations, and chromosome loss. This cytogenetic inhomogeneity suggests that chromosome breaks are common events in L1210 cells. It is assumed that this chromosome fragility is responsible for the enhanced sensitivity to cytotoxic agents in this cell line. Genetically unstable cell populations should have a greater opportunity to generate mutants that confer resistance which will be selected under drug pressure.

Growth Inhibition Tests

The sensitivities of the four cell lines to dFdC and ara-C, expressed as IC_{50} values, are listed in Table 2. The parent murine leukemia cell line L1210 was the most sensitive cell line to both dFdC and ara-C. The ara-C-resistant variant of L1210, L4A6, was 1000- and 2000-fold resistant to dFdC and ara-C, respectively. The other L1210 variants showed a similar resistance profile for ara-C (not shown). Compared

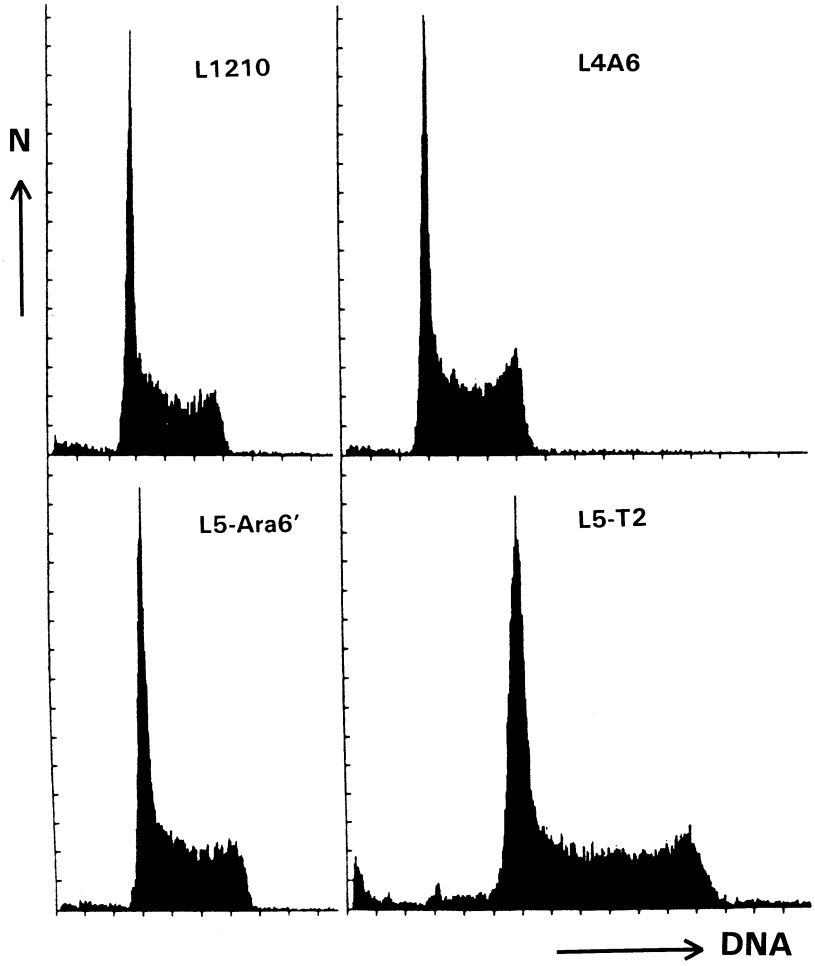

FIG. 1. DNA histograms of the parental cell line L1210 and the ara-C-resistant variants L4A6, L5-ara6′ and L5-T2. No difference in DNA content was observed in L4A6 cells compared to its parental cells.

to L1210, the rat leukemia cell line BCLO was 5-fold less sensitive to dFdC and 17-fold less sensitive to ara-C then L1210. However, the ara-C-resistant variant of BCLO, Bara-C, was 3200-fold and >300-fold resistant to dFdC and ara-C, respectively.

Figure 2A shows representative relative growth inhibition curves of L1210 and L4A6 cells when exposed to either dFdC or ara-C. L1210 cells were highly sensitive to ara-C. The curve of L1210 cells exposed to dFdC was shaped similarly to that for ara-C, but shifted to the lower concentration range. In the resistant L4A6 cells, a similar pattern was found: L4A6 was more sensitive to dFdC than to ara-C. Neither in L1210 or in L4A6 was cell kill observed after exposure to dFdC or ara-C in the concentrations tested. Clonogenic assays (not shown) showed similar resistance patterns.

Figure 2B shows similar curves of BCLO and Bara-C cells. Both cell lines were more sensitive to dFdC than to ara-C. The growth inhibition curves of BCLO and Bara-C exposed to ara-C and dFdC are shaped similarly. No cell kill was achieved following dFdC or ara-C exposure in the concentrations tested.

TABLE 2. IC_{50} values for dFdC and ara-C in the murine leukemia cell line L1210 and the rat leukemia cell line BCLO and their ara-C-resistant variants L4A6 and Bara-C, respectively

Cell line	dFdC	Ara-C
L1210	2.4 ± 0.5	20.3 ± 4.8
L4A6	2700 ± 250	44000 ± 7900
BCLO	12.2 ± 4.4	340 ± 115
Bara-C	39000 ± 14500	>100,000

Values are means ± SEM in nM of 3–7 experiments. Cells were exposed for 48 hr.

Intracellular Ara-C Accumulation

Initial studies on the mechanism of ara-C resistance in the L1210 variants concentrated on ara-C uptake, defined as the sum of ara-C transport into the cell and intracellular accumulation of metabolites, corrected for efflux. In the wild-type L1210 cells, uptake over 2 hr at 25 nM ara-C was 25% of that at 1 μM ara-C, the approximate K_m for ara-C transport. This nonlinear difference possibly reflected both differences in transport rate and metabolism. In the resistant variants, no ara-C uptake was detectable at 25 nM, but at 1 μM ara-C uptake was about 15% of that in L1210 cells.

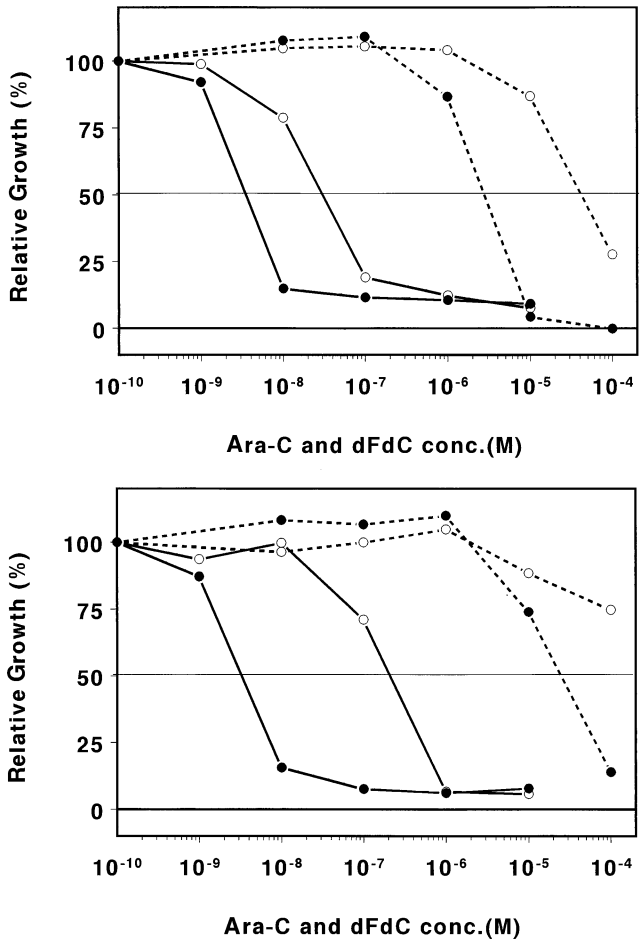

FIG. 2. Representative growth inhibition curves of the cell lines exposed for 48 hr to ara-C (-○-) to dFdC (-●-). (A) L1210 (solid lines) and its ara-C-resistant derivative L4A6 (dotted lines). (B) BCLO (solid lines) and the ara-C-resistant derivative Bara-C (dotted lines).

During the 2-hr exposure period, ara-C can be converted to a phosphorylated form, which is responsible for its retention in the cell. Therefore, it was assumed that ara-C phosphorylation was inhibited in the resistant variants, and subsequent studies concentrated on kinase measurements.

dCK, dCDA, and TK Enzyme Activities

The dCK and dCDA activities of the four leukemic cell lines were measured with dCyd, dFdC, and ara-C as a substrate (Table 3). Completely different patterns were observed for the various cell lines. The murine wild-type L1210 cells showed the highest extent of dCyd and dFdC phosphorylation compared to the other cell lines. dFdC was the best substrate in wild-type L1210. In the ara-C-resistant variant of L1210, L4A6, the phosphorylation was >40- and >45-fold lower with dCyd and dFdC as a substrate, respectively, than in its parental cell line L1210. In the other ara-C-resistant L1210 variants, dCyd phosphoryation was similar to that in L4A6. Surprisingly, in L4A6 cells the dCK activity with ara-C as a substrate was only decreased 3-fold.

TABLE 3. Deoxynucleoside phosphorylation and dCDA activities in the murine leukemia cell line L1210 and the rat leukemia cell line BCLO and their ara-C-resistant variants L4A6 and Bara-C, respectively

Cell line	Substrate	Phosphorylation	dCDA activity
L1210	dCyd	1.57 ± 0.15	ND
	dFdC	2.68 ± 0.52	0.35 ± 0.09
	ara-C	0.27 ± 0.04	ND
L4A6	dCyd	<0.04	ND
	dFdC	<0.06	0.41 ± 0.18
	ara-C	0.09 ± 0.02	ND
BCLO	dCyd	0.55 ± 0.12	ND
	dFdC	0.47 ± 0.02	1.91 ± 0.38
	ara-C	0.26 ± 0.17	ND
Bara-C	dCyd	0.38 ± 0.12	2.27 ± 0.49
	dFdC	<0.01	0.39 ± 0.02
	ara-C	ND	ND

Values are means ± SEM in nM of 3–6 experiments in nmol/hr/10^6 cells (protein content: L1210; 46.9 ± 4.8, L4A6; 56.8 ± 7.2, BCLO; 102.5 ± 16.0, Bara-C; 68.9 ± 9.8 μg/10^6 cells). dCK activity in the other L1210 variants L5-T1, L5-T2, and L5-T3 measured with dCyd as a substrate were comparable with those of L4A6. ND, not detectable.

From the rat leukemia cell lines, the BCLO was less sensitive to dFdC than L1210 cells, which would correlate with a lower kinase activity with both dCyd and dFdC as substrate. However, despite the lower ara-C sensitivity in BCLO compared to L1210, the dCK activity with ara-C as a substrate was equal in both cell lines. The >300-fold ara-C-resistant variant of BCLO, Bara-C, had a comparable rate of dCyd phosphorylation to its parental cell line. However, neither dFdC nor ara-C could be phosphorylated in Bara-C cells.

Although UTP has been reported to be a better phosphate donor for phosphorylation of dCyd, we used ATP as phosphate donor in all experiments. ATP is the most abundant nucleotide in the cell and therefore determines to a larger extent the phosphorylation of dCyd and its analogues. In addition, we wanted to compare different kinase activities with different nucleoside substrates, which subsequently may not prefer UTP as phosphate donor.

Because of the aberrant pattern of deoxynucleoside phosphorylation in the rat cells, indicating the involvement of more kinases, we measured the dCK and TK activities of the rat leukemia cell lines under more specific conditions (Table 4). The phosphorylation of dCyd in BCLO could be inhibited 4.5-fold in the presence of TdR, indicating an important role for TK2 in the phosphorylation of dCyd in this cell line. In the ara-C-resistant cell line Bara-C, the dCyd phosphorylating activity was also inhibited about 4- to 5-fold by TdR, indicating a clear involvement of TK2 in dCyd phosphorylation. The total TK activity, which was measured with TdR as a substrate, was 2-fold higher in BCLO than in Bara-C cells, although the absolute difference was still 1.2 nmol/hr/10^6 cells. The TdR

TABLE 4. dCyd phosphorylation and TK activities in the rat leukemia cell line BCLO and its ara-C-resistant variant Bara-C

Cell line	Substrate/ inhibitor	dCyd phosphorylation	TK activity
BCLO	dCyd	0.55 ± 0.12	
	dCyd+TdR	0.12 ± 0.03	
	TdR		2.52 ± 0.49
	TdR+dCTP		1.11 ± 0.42
Bara-C	dCyd	0.38 ± 0.12	
	dCyd+TdR	0.08 ± 0.04	
	TdR		1.33 ± 0.17
	TdR+dCTP		0.96 ± 0.52

Values are means ± SEM of 3–5 separate experiments in nmol/hr/10^6 cells. In the dCK assay, 5 mM TdR (final concentration) was added to specifically inhibit TK2 activity, with the remaining dCyd phosphorylation considered to be due specifically to dCK. In the TK assay, 5 mM dCTP (final concentration) was added to inhibit TK2 activity in the assay, the difference providing an indication of TK2 activity. The uninhibited value will give an indication of the TK1 activity.

phosphorylation in BCLO was 2-fold lower in the presence of dCTP, which inhibits TK2 but not TK1 under these conditions [29], thus enabling separation to be made between the two enzymes [32]. This indicates an equal TK1 and TK2 activity in this cell line, using TdR as the substrate; TK2 activity may then be estimated to be about 1.3 nmol/hr/10^6 cells. However, in Bara-C the difference in TdR phosphorylation in the presence of dCTP indicated a lower contribution of TK2 activity. All these experiments were performed simultaneously, revealing a similar difference in each experiment. Therefore, TK2 activity can be estimated to be about 0.3–0.4 nmol/hr/10^6 cells. It can be concluded that Bara-C cells have a deficiency in dCK, but contain TK2 activity which is lower than in BCLO cells, but sufficiently high to phosphorylate dCyd.

In neither murine leukemia cell line (L1210 nor L4A6) was dCDA activity found with dCyd or ara-C as a substrate. However, with dFdC as a substrate dCDA activity was comparable in the cell lines, despite the 1000-fold lower sensitivity of L4A6 to dFdC. No dCDA activity was found in the rat leukemia cell line BCLO with dCyd or ara-C as a substrate, but a 5-fold higher dCDA activity was found with dFdC as a substrate compared to L1210 and L4A6 cells. In contrast to L4A6, a high dCDA activity with dCyd as a substrate was found in Bara-C. With dFdC as a substrate, dCDA activities were in the same range as L1210 and L4A6. However, no dCDA activity was found in Bara-C with ara-C as a substrate. Apparently, induction of ara-C resistance in the BCLO cell line also induced dCDA activity, which was associated with aberrant kinetic properties for dCDA. With purified dCDA, we observed that dFdC was a better substrate than dCyd, which in turn was a better substrate than ara-C (data not shown) for dCDA. This pattern was also observed in the L1210 cells. However, in the Bara-C cell line there was a substantial dCDA activity with dCyd, which was even higher than with dFdC, indicating changes in kinetic properties. We did not detect any 2′,2′-difluorodeoxyuridine metabolite in the cells after exposure to dFdC.

dFdCTP and Ara-CTP Accumulation

Accumulation of dFdCTP or ara-CTP was performed with 10 μM dFdC or ara-C, respectively, which are concentrations causing complete growth inhibition in wild-type leukemia cell lines (Table 5). The very sensitive wild-type cell line L1210 accumulated the highest amounts of both dFdCTP and ara-CTP. The dFdC and ara-C-resistant variants of L1210 did not accumulate ara-CTP, while L4A6 cells did not accumulate dFdCTP either. The wild-type rat leukemia cell line BCLO which, compared to L1210 cells, was 6- and 17-fold less sensitive to dFdC and ara-C, respectively, accumulated at least 3-fold less dFdCTP or ara-CTP then L1210 cells. In addition, the 3000-fold dFdC and >300-fold ara-C-resistant variant of BCLO, Bara-C, did not accumulate dFdCTP or ara-CTP.

DISCUSSION

In this paper, we describe the development of an ara-C-resistant variant of the L1210 leukemia cell line, resulting in the cytogenetically stable variant L4A6. This resistance appeared to be related to a dCK deficiency and was associated with cross-resistance to dFdC. Despite aberrant dCyd phosphorylation properties, the ara-C-resistant Bara-C cells also showed cross-resistance to dFdC. Significant differences in ara-C and gemcitabine sensitivity between the murine and rat leukemia cell lines were found. The ara-C-resistant variant L4A6 did not differ in DNA content, doubling time, chromosome number, chromosome stability nor tumorigenicity, but was 1000-fold resistant to ara-C compared to its parental cell line L1210. Because of this cytogenetic similarity, other features which could be responsible for the acquired resistance were studied.

Mechanistic studies concentrated on gemcitabine and ara-C metabolism. The decrease in the total ara-C accumulation in the resistant cell line L4A6 did not appear

TABLE 5. dFdCTP and ara-CTP accumulation in the murine leukemia cell line L1210 and the rat leukemia cell line BCLO and their ara-C-resistant variants L4A6 and Bara-C, respectively

	dFdCTP	Ara-CTP
L1210	368 ± 113	83 ± 30
L4A6	ND	ND
BCLO	113 ± 63	ND*
Bara-C	ND	ND

Cells were exposed to 10 μM dFdC or ara-C for 2 hr. Values are means ± SEM of 3 experiments in pmol/10^6 cells. Ara-CTP accumulation in the other L1210 variants was also not detectable. ND, not detectable.

* After exposure for 4 hr using 4- to 10-fold more cells, ara-CTP accumulation was detectable and was about 30 pmol/10^6 cells; however, in the resistant lines no ara-CTP could yet be measured.

sufficient to explain the drug resistance, since various mechanisms may be responsible for this. Thus, it was concluded that the resistance observed is not due to a reduced drug permeability alone [14, 15].

The dCK activity in both ara-C-sensitive cell lines correlated with the sensitivity of these cell lines to the different drugs. The higher sensitivity to dFdC might be related to its higher affinity for dCK than ara-C (K_m 3.6 and 8.8 μM, respectively) [6]. However, dCK activity with ara-C as a substrate in BCLO was equal to that in L1210, although L1210 was 17-fold more sensitive to ara-C than BCLO. This difference in sensitivity to ara-C cannot be explained by differences in dCDA activity between L1210 and BCLO. With none of the substrates could dCK activity be measured in the ara-C-resistant cell line L4A6. This might clarify its resistance to ara-C and dFdC, but not the difference in sensitivity between ara-C and dFdC. Despite the higher sensitivity of L4A6 to dFdC, the dCDA activity with dFdC as substrate was higher than with ara-C as substrate. The ara-C- and dFdC-specific dCK deficiency in Bara-C might be responsible for its ara-C and dFdC resistance, described previously by Richel *et al.* [23]. The deficiency in specific dCK activity was evident since in Bara-C cells, dFdC could not be phosphorylated, while dCyd phosphorylation catalyzed by dCK was decreased significantly. Apparently, TK2 does not contribute to phosphorylation of ara-C in Bara-C cells, since the IC_{50} values were higher than 100 μM; this is consistent with the low substrate specificity of ara-C for purified TK2 (2–3% of dCyd) [9]. However, the relatively better sensitivity of Bara-C cells to dFdC compared to ara-C may be related to the better substrate specificity of dFdC for purified TK2 (5–10% dCyd).* Even dFdU has a 5–10% substrate specificity of dCyd. Intracellularly, endogenous TdR or dCyd may compete with dFdC conversion; however, in general intracellular deoxynucleoside, concentrations are rather low [33] and possibly insufficient to compete with pharmacological concentrations of drugs such as dFdC [34].

Ara-C resistance due to dCK deficiency has been observed repeatedly in resistant murine leukemia cells [34, 35]. Resistance due to inactivation of ara-C by deaminase activity seems to be very unlikely, since in several studies the presence of this enzyme could not be demonstrated in L1210 cells [36, 37]. Indeed, in the murine leukemia cell lines L1210 and L4A6, no dCDA activity could be detected with dCyd and ara-C as substrates, but with dFdC as a substrate some activity was found. This is probably the result of a very low dCDA activity which cannot be measured with its natural substrate, but is just detectable with dFdC for which it might have a high affinity. In the rat leukemia cell lines, dCDA activities did not clarify the higher sensitivity to dFdC compared to ara-C or the resistance of Bara-C to ara-C. Previous studies showed that tetrahydrouridine (THU), an inhibitor of dCDA, did not result in a significant increase in ara-CTP formation in leukemic cells, suggesting a minor role for dCDA in ara-C resistance in cell lines [38]. There are, however, indications that high dCDA activities are related to poor responses in patients [39].

Since dCK and dCDA activities did not correlate with the higher sensitivity to dFdC than ara-C in all the cell lines tested, accumulation of the active metabolites of both drugs, dFdCTP and ara-CTP, was measured. L1210, the cell line with the highest sensitivity to both dFdC and ara-C, accumulated the highest amounts of both triphosphates, which correlated with its high dCK activity. The dFdC- and ara-C-sensitive wild-type BCLO cells accumulated less dFdCTP and ara-CTP than L1210 cells, in keeping with the lower dCK activity with dFdC as a substrate. The ara-C-resistant cell lines L4A6 and Bara-C did not accumulate dFdCTP or ara-CTP. This resistance appeared to be related to a failure to accumulate sufficient ara-CTP or dFdCTP.

The difference in sensitivity of the cell lines to dFdC and ara-C is only partially clarified by the difference in accumulation of the active metabolites. Despite the similarity of the molecular structures and a common metabolism in activation and inactivation, there are important differences in the metabolism of gemcitabine and ara-C. Previous studies indicated that dFdCTP accumulated at levels up to 20-fold higher than ara-CTP at equimolar concentrations of gemcitabine and ara-C, respectively. Moreover, the elimination pattern of dFdCTP and ara-CTP differed, with dFdCTP being retained longer than ara-CTP. In a human ovarian cancer cell line, dFdCTP was retained for at least 24 hr at the level measured at the time of drug removal [30]. The accumulation of both ara-CTP and dFdCTP is a saturable process, as was shown *in vitro* [6, 30] as well as in leukemic blast cells and peripheral blood lymphocytes from patients [40–42]. In patients, the dFdCTP accumulation was saturated at a higher plasma concentration compared to ara-CTP. Moreover, in contrast to ara-CTP, accumulation of dFdCTP resulted in major changes in (deoxy) ribonucleotide pools. In the human leukemia cell line CCRF-CEM, a considerable decrease in dCTP concentrations was observed, which was related to a marked inhibition of ribonucleotide diphosphate reductase by dFdCDP [43]. Since dCTP is the major feedback inhibitor of dCK, depletion of dCTP would increase the phosphorylation of dFdC by dCK [18]. However, Cory *et al.* did not find a decreased sensitivity to dFdC in a hydroxyurea-resistant variant of L1210 with an increased ribonucleotide reductase activity, although ribonucleotide reductase activity in cell-free extracts prepared from L1210 cells treated with dFdC was reduced by 50% [44]. Both ara-C and dFdC can inhibit DNA synthesis as a result of the incorporation of their triphosphates into DNA, although less dFdC than ara-C was required to achieve the same degree of inhibition [6]. Moreover, the exonuclease activity was unable to excise dFdCMP, whereas ara-CMP was removed at 37% of the excision rate of normal deoxynucleotides. In contrast to

* Wang L, Munch-Petersen B, Herrström Sjöberg A, Bergman T, Hellman U, Jörnvall H, Eriksson S, submitted for publication, 1998.

ara-CTP, dFdCTP is incorporated into RNA as was demonstrated in human ovarian cancer A2780 cells, where almost similar dFdCMP levels were found as in DNA [7]. The different metabolic features of dFdC compared to ara-C may explain the increased sensitivity to dFdC, while the different enzyme make-up of BCLO may explain its relative resistance to dFdC.

Under high-dose ara-C therapy, plasma ara-C levels are 4–10 times in excess of 10 μM ara-C [25, 40], a concentration at which ara-C phosphorylation is saturated [40]. However, both the murine and rat ara-C-resistant leukemia cell lines have IC_{50} values similar to or higher than these ara-C plasma levels. The peak plasma concentrations of dFdC at the maximum tolerated dose in a twice weekly schedule (5700 mg/m^2) were 400–500 μmol/L, which is 170- and 12-fold higher then the IC_{50} value of the ara-C-resistant cell lines L4A6 and Bara-C, respectively [25, 41]. In addition, at the lower standardly used dose of 1000–1250 mg/m^2, peak plasma concentrations are higher than IC_{50} values of dFdC in the resistant cell lines. dFdC can also be given at prolonged infusions at this dose, while recent studies have redefined the maximum tolerated dose of dFdC at more than 2000 mg/m^2. The higher plasma levels of dFdC which can be reached in these schedules in combination with the high activity of dFdC in ara-C-sensitive and-resistant leukemia cell lines suggest the possible worth of dFdC in the treatment of leukemia. After the initial discontinuation of dFdC studies in leukemia [41], new clinical studies in leukemia with prolonged infusion of gemcitabine were initiated. Considering the activity in previous studies with low doses and shorter infusions, it is not unlikely that gemcitabine at this schedule will show activity and might possibly be considered for treatment of AML in place of ara-C, similar to the replacement of daunomycin by idarubicin [45].

In conclusion, gemcitabine is more active than ara-C in the ara-C-sensitive and -resistant murine and rat leukemia cell lines tested. This higher sensitivity does not correlate with dCK and dCDA activities and is only partially clarified by the accumulation of triphosphates of the drugs. Probable differences in the inhibition of DNA synthesis by dFdC and ara-C, the incorporation of gemcitabine into RNA, and the self potentiation of dFdC as a result of changes in (deoxy) ribonucleotide pools might be an explanation for the higher sensitivity to dFdC than to ara-C of the cell lines tested.

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