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Role of Deoxycytidine Kinase in the Inhibitory Activity of 5-Substituted 2′-Deoxycytidines and Cytosine Arabinosides on Tumor Cell Growth

JAN BALZARINI AND ERIK DE CLERCQ

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

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SUMMARY

A number of 5-substituted derivatives of dCyd and 1-β-D-arabinofuranosylcytosine (araC) have been evaluated for their inhibitory effects on the growth of three murine leukemia cell lines (L1210/0, L1210/BdUrd, and L1210/araC). The L1210/BdUrd and L1210/araC cell lines were selected from the parental L1210/0 cell line by their ability to grow at high concentrations of 5-bromo-2′-deoxyuridine and araC, respectively; the L1210/BdUrd cell line was deficient in dThd kinase activity, whereas the L1210/araC cell was deficient in dCyd kinase activity. The most effective inhibitors of L1210/0 cell proliferation were 5-fluoro-dCyd, araC, and 5-fluoro-araC. Their 50% inhibitory dose fell within the 0.001–0.015 μg/ml range. The 5-substituted araC analogues were much less inhibitory for L1210/araC cells but equally inhibitory for L1210/BdUrd as for the parental L1210/0 cell line. The role of dCyd kinase in the antitumor activity of the dCyd and araC analogues was further assessed by kinetic studies with dCyd kinase extracted from L1210/0 cells. All dCyd and araC analogues caused a competitive inhibition of dCyd kinase, the most potent inhibitor being 5-fluoro-dCyd (K_i/K_m value 0.24). The K_m of dCyd kinase from L1210/0 cells for dCyd was 23.1 μM as compared with 50 μM for araC. These values were increased to 53 and 182 μM, respectively, for the dCyd kinase isolated from L1210/araC cells.

INTRODUCTION

The antitumor cell activity of 5-substituted 2′-deoxyuridines such as 5-fluoro-dUrd, 5-trifluoromethyl-dUrd, 5-ethynyl-dUrd, and 5-formyl-dUrd depends to a large extent on phosphorylation by the dThd kinase (EC 2.7.1.21) of the tumor cells (1). Upon conversion to their 5′-monophosphate form these compounds would interfere with dTMP synthetase, and their anti-tumor cell activity would ultimately result from a shutoff of DNA synthesis (2). In contrast to the dUrd derivatives, dCyd analogues such as araC,[1] are phosphorylated by dCyd kinase (EC 2.7.1.74), and resistance of tumor cell lines to the growth-inhibiting effects of araC has been associated with decreased levels of dCyd kinase (3, 4).

We have now evaluated the role of dCyd kinase in the inhibitory effects of a variety of dCyd analogues, including 5-fluoro-dCyd, 5-fluoro-araC, and several other 5-substituted dCyd and araC derivatives, on the growth of murine leukemia (L1210) cells.

The role of phosphorylation by dCyd kinase in the cytotoxic activity of these compounds was assessed by (a) including among the tumor cell lines tested a dCyd kinase-deficient mutant L1210 cell line [L1210/araC, selected by its ability to grow in the presence of araC (1 μg/ml)], and (b) measuring the K_i (apparent inhibitory constant) of the test compounds for the isolated L1210 dCyd kinase.

MATERIALS AND METHODS

Cells. Murine leukemia L1210 cells were grown in 75 cm^2 tissue culture flasks (Falcon 3024F; Becton, Dickinson France S.A., Grenoble, France) in Eagle's minimal essential medium, supplemented with 10% (v/v) inactivated fetal calf serum (GIBCO Bio-Cult, Glasgow, Scotland) and 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland).

Compounds. Pyruvate kinase, phosphoenolpyruvate, and ATP were obtained from Sigma Chemical Company (St. Louis, Mo.). 2-Mercaptoethanol was obtained from Fluka AG (Bucks, Switzerland).

The test compounds and their sources were as follows: dCyd (Sigma Chemical Company), 5-fluoro-dCyd (courtesy of H. J. Scholer and A. Polak, F. Hoffmann-La Roche & Company, Basle, Switzerland), 5-chloro-dCyd (Calbiochem-Behring Corporation, Lucerne, Switzerland), 5-bromo-dCyd (Sigma Chemical Company), 5-iodo-dCyd (Serva Feinbiochemica, Heidelberg, Germany), 5-methyl-dCyd (Calbiochem-Behring Corporation), 5-propyl-dCyd (see ref. 5), (*E*)-5-(2-bromovinyl)-dCyd (see ref. 6), araC (Upjohn Company, Puurs, Belgium), 5-fluoro-araC (courtesy of P. F. Torrence, Na-

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[1] The abbreviation used is: araC, 1-β-D-arabinofuranosylcytosine.

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TABLE 1

Inhibitory activity of 5-substituted 2′-deoxycytidines and cytosine arabinosides on murine L1210 cell growth and DNA synthesis

Compound	ID_{50} for cell growth upon addition of				ID_{50} for DNA synthesis as monitored by incorporation of		
	As such	dUrd (125 μg/ml)[a]	dThd (5 μg/ml)[a]	dCyd (500 μg/ml)[a]	[*methyl*-^{3}H]dThd	[1′,2′-^{3}H]dUrd	[5-^{3}H]dCyd
	μg/ml				μg/ml		
5-Fluoro-dCyd	0.001	0.023	≥1000	0.065	>1000	0.0009	0.0008
5-Chloro-dCyd	>1000	>1000	>1000	>1000	114.5	6.8	515
5-Methyl-dCyd	>1000	>1000	>1000	>1000	102	29.2	100
(*E*)-5-(2-Bromovinyl)-dCyd	30[b]	127[b]	519[b]	129[b]	>1000[b]	21[b]	27[b]
AraC	0.010	0.013	0.010	32.0	0.0361	0.0135	0.0816
5-Fluoro-araC	0.015	0.020	0.019	41.0	0.0447	0.0184	0.0657
5-Chloro-araC	2.4	3.8	4.6	>1000	41	6.1	7.8
5-Chloromercuri-araC	0.109	0.092	0.147	19.7	0.660	0.282	9.03
5-Propyl-araC	>100	>100	>100	>100	>100	>100	>100
5-Isopropyl-araC	>100	>100	>100	>100	>100	>100	>100

[a] Maximal concentrations of dUrd, dThd, and dCyd that were themselves not inhibitory to L1210 cell growth.
[b] Data taken from ref. 8.

tional Institutes of Health, Bethesda, Md.), 5-chloro-araC (courtesy of P. F. Torrence), 5-chloromercuri-araC (Calbiochem-Behring Corporation), 5-propyl-araC (courtesy of D. Shugar, Polish Academy of Sciences, Warszawa, Poland), 5-isopropyl-araC (courtesy of D. Shugar), dUrd (Calbiochem-Behring Corporation), 5-fluoro-dUrd (Aldrich Chemical Company, Milwaukee, Wisc.), 5-chloro-dUrd (Calbiochem-Behring Corporation), 5-bromo-dUrd (Sigma Chemical Company), 5-iodo-dUrd (Sigma Chemical Company), dThd (Sigma Chemical Company), 5-propyl-dUrd (see ref. 7), (*E*)-5-(2-bromovinyl)-dUrd (see ref. 6).

Radiochemicals. The radiolabeled nucleosides [1′,2′-^{3}H]dUrd (specific radioactivity 31 Ci/mmole), [5-^{3}H]dCyd (specific radioactivity 22 Ci/mmole), and [5-^{3}H]araC (specific radioactivity 15.5 Ci/mmole) were obtained from the Radiochemical Centre (Amersham, England), whereas [*methyl*-^{3}H]dThd (specific radioactivity 47 Ci/mmole) was obtained from the Institute of Radio-Elements (IRE, Fleurus, Belgium).

Selection of L1210/BdUrd and L1210/araC cells. The parental L1210/0 cells were seeded into tissue culture dishes (Falcon 3002F, Becton Dickinson France S.A., Grenoble, France) at a density of 2×10^5 cells/dish in the presence of 6 ml of growth medium supplemented with 5-bromo-dUrd (25 μg/ml) or araC (10 ng/ml). After 2 or 3 days, the cells were distributed to three new tissue culture dishes, again in the presence of 6 ml of growth medium plus 5-bromo-dUrd (25 μg/ml) or araC (10 ng/ml). The concentrations of 5-bromo-dUrd and araC were then gradually increased at each cell passage until they reached 260 μg/ml for 5-bromo-dUrd and 1 μg/ml for araC. We were able to select the BdUrd-resistant and araC-resistant sublines (designated L1210/BdUrd and L1210/araC, respectively) after about 10–15 passages.

The L1210/BdUrd and L1210/araC sublines were maintained in normal growth medium under the conditions used for the parental L1210/0 cells. No selective pressure was required for stability of the mutant cell lines. However, these cell lines were not cloned, and

TABLE 2

Inhibitory effects of 5-substituted 2′-deoxycytidines and cytosine arabinosides on the growth of L1210/0, L1210/BdUrd, and L1210/araC cells

Compound	ID_{50}			ID_{50} L1210/BdUrd/ ID_{50} L1210/0	ID_{50} L1210/araC/ ID_{50} L1210/0
	L1210/0[a]	L1210/BdUrd[a]	L1210/araC[a]		
	μg/ml				
dCyd	>1000	>1000	>1000	—	—
5-Fluoro-dCyd	0.001 (±0.0006)	0.017 (±0.006)	0.025 (±0.0007)	17.0	25.0
5-Chloro-dCyd	>1000	>1000	>1000	—	—
5-Bromo-dCyd	>1000[b]	>1000	>1000	—	—
5-Iodo-dCyd	>1000[b]	>1000	>1000	—	—
5-Methyl-dCyd	>1000	>1000	>1000	—	—
5-Propyl-dCyd	>1000[b]	>1000	>1000	—	—
(*E*)-5-(2-Bromovinyl)-dCyd	30.0 (±4.67)[b]	4.78 (±0.66)	55.7 (±4.22)	0.16	1.86
AraC	0.010 (±0.003)	0.012 (±0.011)	4.73 (±0.36)	1.20	473
5-Fluoro-araC	0.015 (±0.006)	0.012 (±0.0013)	4.23 (±0.24)	0.80	282
5-Chloro-araC	2.4 (±1.10)	3.61 (±0.29)	>1000	1.50	>417
5-Chloromercuri-araC	0.109 (±0.093)	0.059 (±0.001)	27 (±1.0)	0.54	248

[a] L1210/BdUrd and L1210/araC are murine leukemia L1210 cell lines selected from the parental L1210/0 cell line for their ability to grow in the presence of 5-bromo-dUrd (260 μg/ml) and araC (1 μg/ml), respectively.
[b] Data taken from ref. 8.

therefore may have consisted of different phenotypic variants.

Inhibition of tumor cell growth. All assays were performed in Linbro microplates. The cells were suspended in growth medium and added to the microplate wells at a density of 5×10^4 cells/well in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 42–48 hr at 37° in a humidified, CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter (Coulter Electronics Ltd., Harpenden Herts, England). The ID_{50} (50% inhibitory dose) was defined as the concentration of compound that reduced the number of living cells by 50%.

Inhibition of [methyl-^{3}H]dThd, [1′,2′-^{3}H]dUrd, and [5-^{3}H]dCyd incorporation into cellular DNA. The incorporation of [*methyl*-^{3}H]dThd, [1′,2′-^{3}H]dUrd, and [5-^{3}H]dCyd into cellular DNA was measured in Linbro microplates (Model FB-48-TC, Linbro Chemical Company, New Haven, Conn.). To each well were added 10^5 L1210 cells and either 5.31 pmoles (0.25 μCi) of [*methyl*-^{3}H]dThd or 8.06 pmoles (0.25 μCi) of [1′,2′-^{3}H]dUrd or 11.3 pmoles (0.25 μCi) of [5-^{3}H]dCyd. The cells were allowed to proliferate for 20 hr at 37° in a humidified, CO_2-controlled atmosphere. At the end of this incubation period, the contents of the wells (200 μl) were brought onto 25-mm glass-fiber filters (Type A/E, Gelman Instrument Company, Ann Arbor, Mich.), mounted on a Millipore 3025 sampling manifold apparatus. The filters were washed twice with cold phosphate-buffered saline, twice with cold 10% trichloroacetic acid, twice with cold 5% trichloroacetic acid, once with cold ethanol, and once with cold ether. The filters were then allowed to dry for 10 min at 60° and assayed for radioactivity in a toluene-based scintillant.

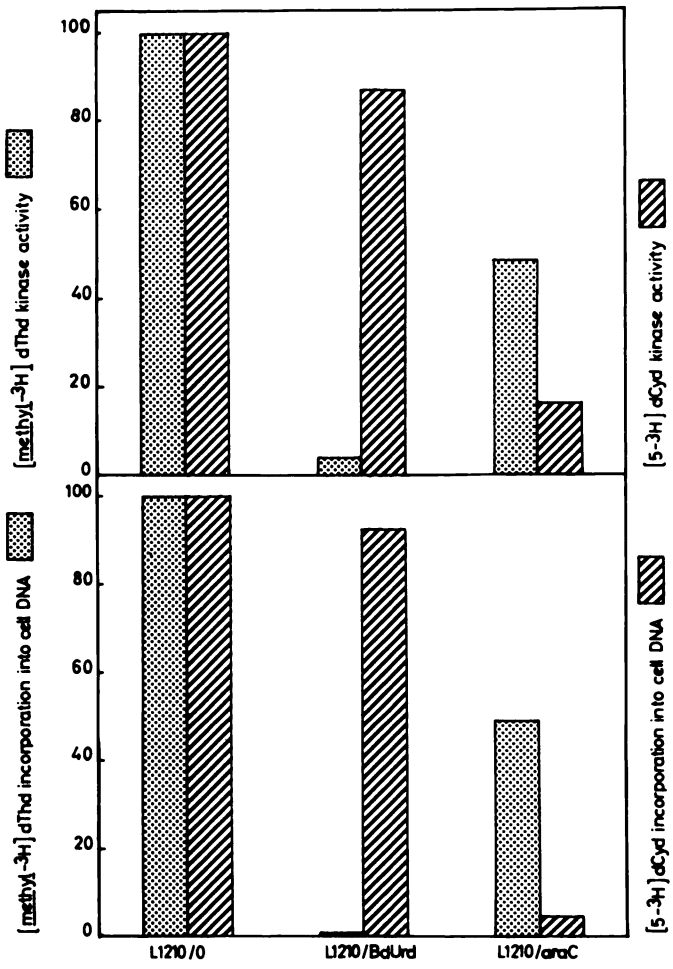

FIG. 1. *dThd kinase and dCyd kinase activity in cell-free extracts and dThd and dCyd incorporation into DNA of L1210/0 cells, L1210/BdUrd cells, and L1210/araC cells*

TABLE 3

Inhibitory effects of 5-substituted 2′-deoxyuridines on the growth of L1210/0 and L1210/araC cells

Compound	ID_{50}		ID_{50} L1210/araC+ ID_{50} L1210/0
	L1210/0[a]	L1210/araC[a]	
	μg/ml		
dUrd	330 (±20.0)	>1000	>3.03
5-Fluoro-dUrd	0.001 (±0.001)[b]	0.002 (±0.0004)	2
5-Chloro-dUrd	23.9 (±6.06)	280 (±70.0)	11.72
5-Bromo-dUrd	26.0 (±4.7)[b]	333 (±28.9)	12.81
5-Iodo-dUrd	138 (±10)	178.4 (±0.50)	1.29
5-Methyl-dUrd (dThd)	12.5 (±3.5)	>1000	>80
5-Propyl-dUrd	>1000[b]	>1000	—
(*E*)-5-(2-Bromovinyl)-dUrd	26.9 (±1.9)[b]	25.7 (±2.90)	0.96

[a] L1210/araC is a murine leukemia L1210 cell line selected from the parental L1210/0 cell line for its ability to grow in the presence of araC (1 μg/ml).
[b] Data taken from ref. 9.

dCyd and dThd kinase assays. The cell pellets were washed twice with cold 0.9% NaCl/0.01 M Tris-HCl buffer (pH 8.0) and once with cold 0.05 M Tris-HCl (pH 8.0) containing 0.02 M β-mercaptoethanol. The cells were then suspended in the latter buffer at a density of 10^8 cells/ml, sonicated twice for 10 sec, cleared by centrifugation at 100,000 × *g* for 45 min, and stored in aliquots at −70°.

The cell extracts were assayed for dCyd or dThd kinase activity in a standard reaction mixture containing 5 mM

TABLE 4

Inhibition of L1210/0 dCyd kinase by 5-substituted 2′-deoxycytidines and cytosine arabinosides

Compound	K_i/K_m[a]
5-Fluoro-dCyd	0.24
5-Chloro-dCyd	7.31
5-Bromo-dCyd	45.5
5-Iodo-dCyd	46.9
5-Methyl-dCyd	16.5
(*E*)-5-(2-Bromovinyl)-dCyd	≫20.0[b]
5-Propyl-dCyd	≫39.8[b]
AraC	44.8
5-Fluoro-araC	7.98
5-Chloro-araC	≫50[b]
5-Chloromercuri-araC	107
dCMP	0.65

[a] K_m values for the individual experiments ranged from 18.7 μM to 30.3 μM. The average K_m value was 23.1 ± 2.6 μM. The inhibition was competitive with respect to dCyd for all dCyd and araC analogues tested.
[b] These K_i/K_m values correspond to the ratio of the highest concentration tested to the K_m value for the individual experiment.

ATP, 5 mM $MgCl_2 \cdot 6H_2O$, 9 mM KF, 5 mM phosphoenolpyruvate, 5 μg of pyruvate kinase, 10 mM β-mercaptoethanol, 0.2 mM (0.1 μCi) [5-^{3}H]dCyd or [*methyl*-^{3}H]dThd in the absence or presence of varying amounts of inhibitor (as indicated in the legends to the figures), and 10 μl of cell extract in a total volume of 40 μl of Tris-HCl 0.05 M (pH 8.0). The reaction mixture was incubated at 37° for 15 min and the reaction was terminated by the addition of 75 μl of ice-cold 0.05 M Tris-HCl buffer (pH 8.0). After boiling for 2 min, the mixture was applied onto DE81 discs and washed with 1 mM NH_4OOCH (pH 8.2), ethanol, and ether. The filters were then assayed for radioactivity in a toluene-based scintillant.

RESULTS AND DISCUSSION

Inhibitory effects of 5-substituted dCyd and araC derivatives on L1210 cell growth and DNA synthesis. From a series of 5-substituted dCyd and araC derivatives that were examined for their inhibitory effects on L1210 cell proliferation, 5-fluoro-dCyd emerged as the most potent inhibitor (Table 1). Its ID_{50} was 1 ng/ml. The same ID_{50} was noted previously for 5-fluoro-dUrd (9). Of the other 2′-deoxycytidines, only (*E*)-5-(2-bromovinyl)-dCyd showed some inhibitory activity against L1210 cell growth. Several other dCyd analogues (i.e., 5-bromo-dCyd, 5-iodo-dCyd, and 5-propyl-dCyd) were inactive against L1210 cell proliferation, even if assayed at a concentration as high as 1000 μg/ml (8). For (*E*)-5-(2-bromovinyl)-dCyd, the ID_{50} was also similar to that previously noted for its dUrd counterpart (9). With ID_{50} values of 10 ng/ml and 15 ng/ml, araC and 5-fluoro-araC were the most potent inhibitors among the araC class. Other araC derivatives, i.e., 5-propyl-araC and 5-isopropyl-araC, failed to inhibit L1210 cell growth, even at 100 μg/ml, the highest concentration tested (Table 1).

The inhibitory effect of 5-fluoro-dCyd on tumor cell growth was completely reversed by dThd but not by dUrd or dCyd. Furthermore, 5-fluoro-dCyd strongly inhibited the incorporation of dUrd and dCyd into DNA but did not inhibit the incorporation of dThd (Table 1). It has been postulated previously (9) that dUrd derivatives, which are far more inhibitory for dUrd than for dThd incorporation and whose tumor cell-inhibiting effects are more readily reversed by dThd than by dUrd, owe their antitumor activity to a selective inhibition of dTMP synthetase. According to the results obtained in Table 1, this premise also holds for 5-fluoro-dCyd, and, to a lesser extent, (*E*)-5-(2-bromovinyl)-dCyd. To act as

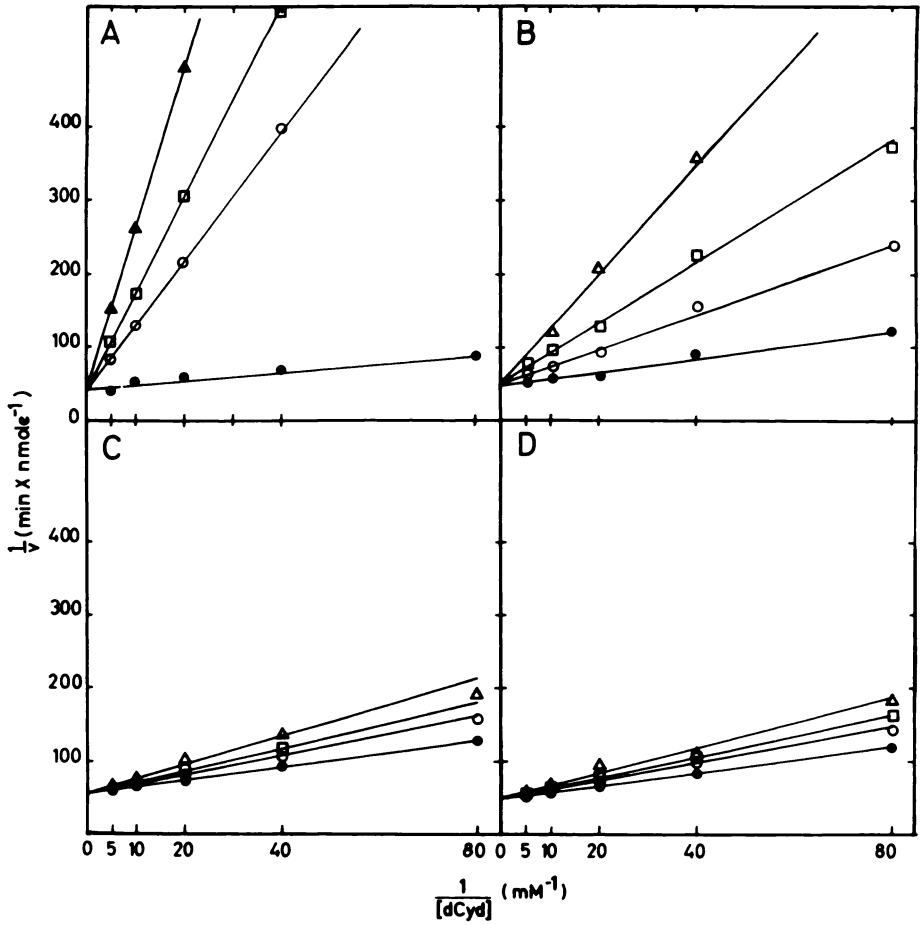

FIG. 2. *Double-reciprocal plots for inhibition of L1210/0 dCyd kinase by 5-fluoro-dCyd (A), 5-chloro-dCyd (B), 5-bromo-dCyd (C), and 5-iodo-dCyd (D)*

Inhibitor concentrations: none (●), 50 μM (○), 100 μM (□), and 200 μM (△) for 5-fluoro-dCyd; none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-chloro-dCyd; none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-bromo-dCyd; none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-iodo-dCyd.

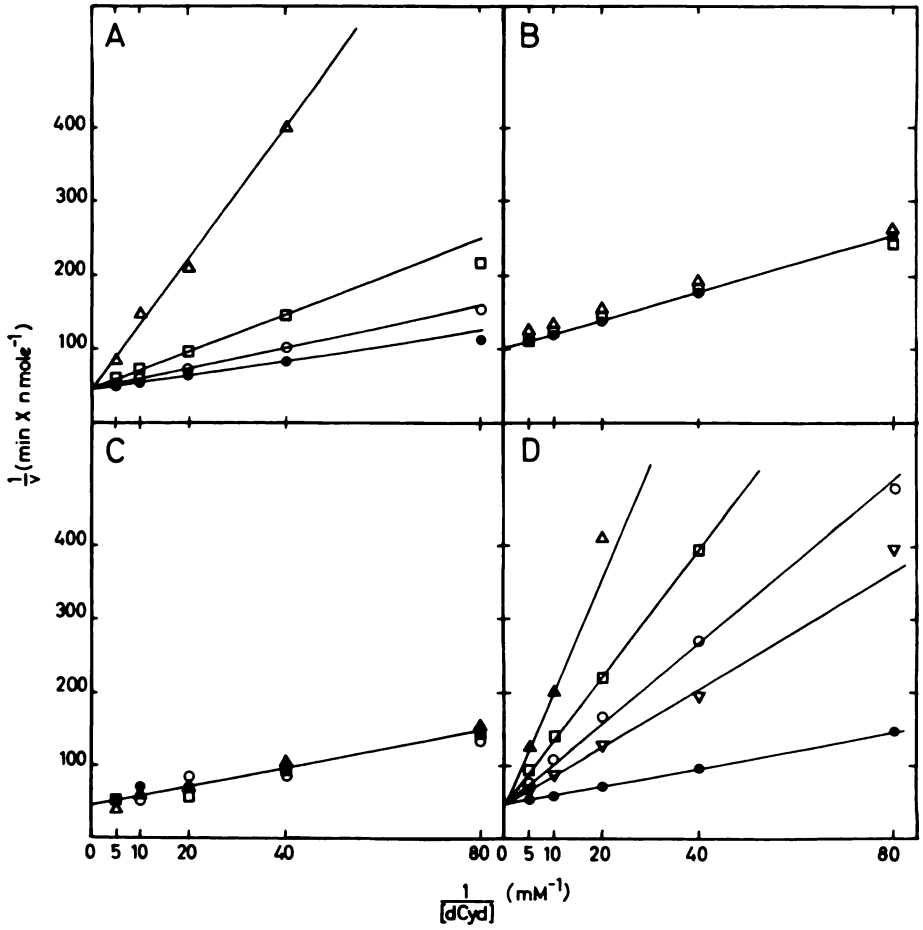

FIG. 3. *Double-reciprocal plots for inhibition of L1210/0 dCyd kinase by 5-methyl-dCyd (A), 5-propyl-dCyd (B), (E)-5-(2-bromovinyl)-dCyd (C), and dCMP (D)*

Inhibitor concentrations: none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-methyl-dCyd; none (●), 500 μM (□), and 1000 μM (△) for 5-propyl-dCyd; none (●), 100 μM (○), 200 μM (□), and 400 μM (△) for (*E*)-5-(2-bromovinyl)-dCyd; none (●), 25 μM (▽), 50 μM (○), 100 μM (□), and 200 μM (△) for dCMP.

inhibitors of dTMP synthetase, these dCyd derivatives should first be deaminated either at the nucleoside or nucleoside 5′-monophosphate level.

Unlike 5-fluoro-dCyd and (*E*)-5-(2-bromovinyl)-dCyd, which inhibited the incorporation of dUrd and dCyd to the same extent, several other dCyd derivatives, in particular 5-bromo-dCyd and 5-iodo-dCyd, proved much more inhibitory for dUrd than for dCyd incorporation. Possible reasons for the anomalous behavior of 5-bromo-dCyd and 5-iodo-dCyd have been discussed previously (8).

The inhibitory effects of the 5-substituted araC derivatives on L1210 cell growth could, to a significant degree, be overcome by dCyd but not by dUrd or dThd. Such a result might be expected if the araC derivatives competed with dCyd for phosphorylation by dCyd kinase, and, as shown below, this assumption proved to be correct.

Inhibitory effects of 5-substituted dCyd and araC derivatives on the growth of L1210/araC and L1210/BdUrd cells. The 5-substituted dCyd and araC derivatives showed marked differences in their inhibitory effects on L1210/0 and L1210/araC cells (Table 2). For all 5-substituted dCyd and araC derivatives that showed ID_{50} values below 1000 μg/ml, except for (*E*)-5-(2-bromovinyl)-dCyd, the ID_{50} for L1210/araC cells was significantly higher than the ID_{50} for the parent L1210/0 cells. For 5-fluoro-dCyd the difference in ID_{50} for the mutant and the parental cell line was 25-fold, whereas the ID_{50} of (*E*)-5-(2-bromovinyl)-dCyd for the mutant cell line was only 1.86 times higher than the ID_{50} for the parental cell line. For araC, 5-fluoro-araC, 5-chloro-araC, and 5-chloromercuri-araC, the ratio of ID_{50} for L1210/0 cells to ID_{50} for L1210/araC cells was between 250 and 500 (Table 2).

It was unequivocally proven that L1210/araC cells were deficient in dCyd kinase activity first (a) by measuring the incorporation of [5-^{3}H]dCyd into cellular DNA, and second (b) by determining the dCyd kinase activity in cell-free extracts (Fig. 1). For L1210/araC cells, [5-^{3}H]dCyd incorporation into DNA was decreased to 4.5% of the value obtained for L1210/0 cells (14.96 ± 3.14 pg/10^5 cells/hr). Concomitantly, the dCyd kinase activity measured in extracts from L1210/araC cells decreased to 16.2% of the value obtained for L1210/0 cell extracts (16.8 ± 1.71 nmoles/mg of protein per hour) (Fig. 1).

The dCyd and araC derivatives have also been evaluated against another L1210 mutant cell line (L1210/BdUrd) that was previously shown to be deficient in

dThd kinase activity (1). All araC derivatives inhibited the growth of L1210/BdUrd cells at ID_{50} values that were comparable with the ID_{50} values required for inhibition of L1210/0 cell growth. However, 5-fluoro-dCyd showed a 17-fold higher ID_{50} for L1210/BdUrd than for L1210/0 cells (Table 2). (*E*)-5-(2-Bromovinyl)-dCyd showed a 6-fold higher ID_{50} for L1210/0 cells than for L1210/BdUrd cells. Similarly, (*E*)-5-(2-bromovinyl)-dUrd was about 20 times more inhibitory for L1210/BdUrd than for L1210/0 cells (1).

The decreased inhibitory effect of 5-fluoro-dCyd on L1210/BdUrd cell growth suggests that, for its activation, 5-fluoro-dCyd partially depends on the dThd kinase activity of the cell, which, in turn, implies that 5-fluoro-dCyd is deaminated to 5-fluoro-dUrd after it has been taken up by the cells.

When the dUrd counterparts of the 5-substituted dCyd analogues listed in Table 2 were examined for their inhibitory effects on L1210/araC cell growth, we expected them to be equally inhibitory for L1210/araC as for L1210/0 cells. This proved to be the case for several dUrd derivatives [i.e., 5-fluoro-dUrd, 5-iodo-dUrd, and (*E*)-5-(2-bromovinyl)-dUrd (Table 3)] and for several other dUrd derivatives (i.e., 5-trifluoromethyl-dUrd, 5-nitro-dUrd, 5-formyl-dUrd, 5-ethynyl-dUrd, 5-azidomethyl-dUrd, . . .) (data not shown). However, dUrd, dThd, 5-chloro-dUrd, and 5-bromo-dUrd exhibited considerably higher ID_{50} values for L1210/araC than for L1210/0 cells (Table 3). It is not immediately clear why these dUrd derivatives should be less effective against the L1210/araC cell line. Possibly dUrd, dThd, 5-chloro-dUrd, and 5-bromo-dUrd might be recognized as substrates by the cellular dCyd kinase. This possibility could be ruled out, however, since the K_i values of dCyd kinase for the 5-substituted dUrd analogues were invariably higher than 1 mM (data not shown). A second possibility is that the L1210/araC cell dThd kinase had been altered in its substrate specificity for dUrd, dThd, 5-chloro-dUrd, and 5-bromo-dUrd. However, there was no difference in the K_m for dThd of the dThd kinase from L1210/araC cells and the dThd kinase from L1210/0 cells. Moreover, 5-bromo-dUrd, which proved more than 10 times less inhibitory for L1210/araC cells than for L1210/0 cells, showed an identical K_i/K_m value with both dThd kinases. These findings argue against an alteration of the substrate specificity of the L1210/araC cell dThd kinase. As a third possibility we may envisage the reduction in dThd kinase activity noted for the L1210/araC cell line (Fig.

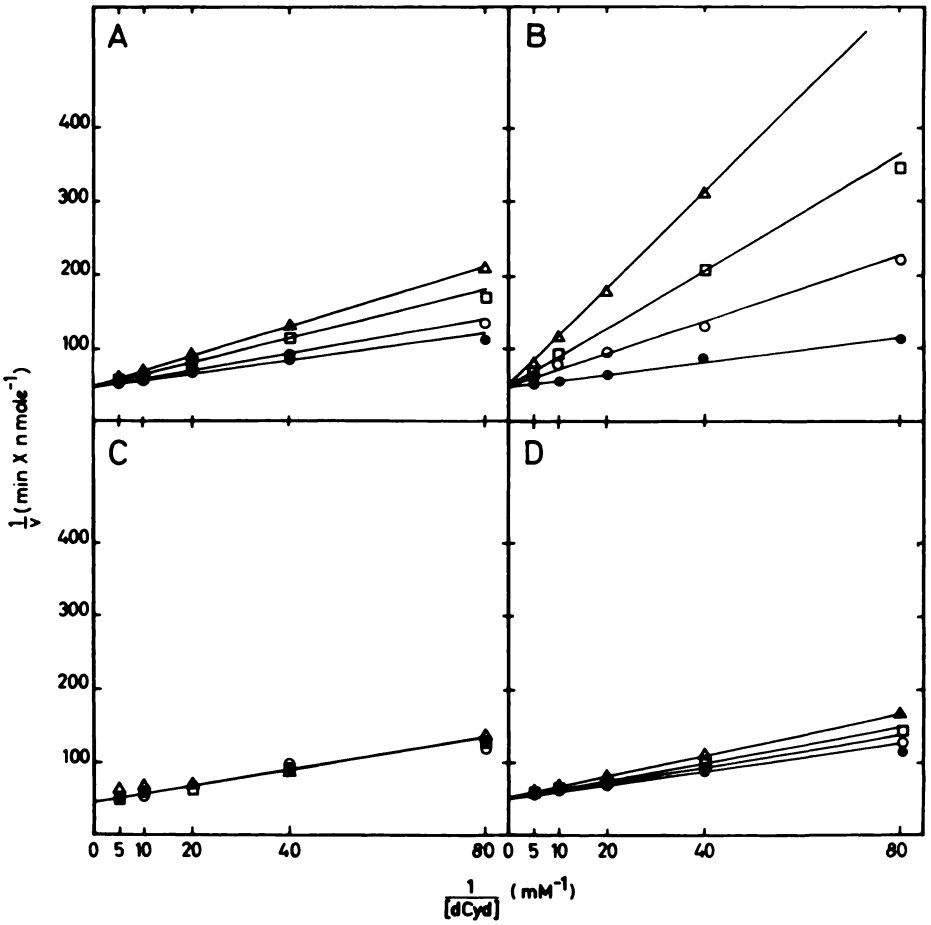

FIG. 4. *Double-reciprocal plots for inhibition of L1210/0 dCyd kinase by araC (A), 5-fluoro-araC (B), 5-chloro-araC (C), and 5-chloromercuri-araC (D)*

Inhibitor concentrations: none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for araC; none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-fluoro-araC; none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-chloro-araC; none (●), 200 μM (○), 500 μM (□), and 1000 μM (△) for 5-chloromercuri-araC.

1). However, this reduction was only 50%, and it is difficult to conceive why this diminished dThd kinase activity would affect only the anti-tumor cell activity of dThd, dUrd, 5-chloro-dUrd and 5-bromo-dUrd, and not of the other dUrd analogues [5-iodo-dUrd, 5-fluoro-dUrd and (*E*)-5-(2-bromovinyl)-dUrd].

Inhibition of L1210 dCyd kinase by 5-substituted dCyd and araC derivatives. The K_i/K_m values of L1210/0 dCyd kinase for the 5-substituted dCyd and araC derivatives are presented in Table 4. The K_m value of the enzyme for dCyd was 23.1 ± 2.6 μM. The most potent inhibitor of dCyd kinase was 5-fluoro-dCyd (K_i/K_m = 0.24). 5-Chloro-dCyd and 5-fluoro-araC were about 30 times less inhibitory. Whereas 5-methyl-dCyd showed a K_i/K_m value of 16.5, 5-bromo-dCyd, 5-iodo-dCyd, and araC showed a relatively low affinity for the enzyme (K_i/K_m = 45). 5-Propyl-dCyd, (*E*)-5-(2-bromovinyl)-dCyd, and 5-chloro-araC did not show any affinity for dCyd kinase, even if tested at a concentration as high as 1 mM). In contrast, dCMP exerted product inhibition with a K_i/K_m value of 0.65. For all dCyd and araC analogues that were inhibitory to dCyd kinase, the type of inhibition appeared to be competitive with respect to dCyd, as attested by Lineweaver-Burk plots (Figs. 2, 3, and 4).

The decreased inhibitory activity of 5-fluoro-dCyd and the 5-substituted araC derivatives toward L1210/araC cell growth may be ascribed not only to a decreased activity of dCyd kinase, but also to an alteration in the kinetic properties of the enzyme. Indeed, when the substrate specificities of [5-^{3}H]dCyd and [5-^{3}H]araC were measured for the L1210/0 and L1210/araC dCyd kinase, some differences were noted between the K_m values of these enzymes. The K_m values of L1210/0 dCyd kinase for dCyd and araC were 24 and 50 μM, respectively. The corresponding K_m values for the L1210/araC dCyd kinase were 53 and 182 μM. Thus, the K_m values of the dCyd kinase from the araC-resistant L1210 cell line were about 3 times higher than those of the parental dCyd kinase. A similar observation has been made for an araC-resistant fibrosarcoma cell line (10). The dCyd kinase from L1210/BdUrd cells showed K_m values for dCyd and araC that were quite similar to the K_m values of the dCyd kinase from L1210/0 cells (data not shown). The K_m value that we found for araC (50 μM) with the dCyd kinase from L1210/0 cells corresponded well with the K_m values found by Meyers and Kreis (11) for murine neoplasm P815 cells (30.2 μM), by Schrecker (3) for murine leukemia L1210 cells (25 μM), and by Lee *et al.* (10) for murine leukemia L51784 cells and hamster fibrosarcoma A(T_1)C1-3 cells (40 μM).

CONCLUSION

In general, 5-substituted dCyd derivatives (i.e., 5-chloro-dCyd, 5-bromo-dCyd, 5-iodo-dCyd, 5-nitro-dCyd, and 5-ethynyl-dCyd) are less cytotoxic to L1210 cells than the corresponding 5-substituted dUrd derivatives (8, 9). Two exceptions to this rule are 5-fluoro-dCyd and (*E*)-5-(2-bromovinyl)-dCyd, which proved as active against L1210 cell growth as their dUrd counterparts. The cytotoxicity of the latter two dCyd analogues may at least partially be attributed to an inhibitory activity at the thymidylate synthetase reaction. To achieve this inhibitory effect, the dCyd analogues probably must first be deaminated either at the nucleoside or at the nucleotide level.

5-Fluoro-dCyd and 5-substituted araC derivatives (i.e., 5-fluoro-araC, 5-chloro-araC, and 5-chloromercuri-araC) are highly dependent on phosphorylation by the cellular dCyd kinase for their inhibitory activity on L1210 cell growth: (a) they are considerably less active against a mutant, dCyd kinase-deficient, L1210/araC cell line that has been selected from the parent L1210/0 cell line for its ability to grow in the presence of high concentrations of araC, and (b) they show a marked inhibitory effect on dCyd kinase isolated from L1210/0 cells; this inhibition is competitive with respect to the natural substrate, dCyd.

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Send reprint requests to: Dr. Erik De Clercq, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.