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Phosphorylation of 5-Halogenated Deoxycytidine Analogues by Deoxycytidine Kinase

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SUMMARY

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The phosphorylation of 5-substituted deoxycytidine analogues by deoxycytidine kinase was studied in extracts of mouse and human lymphoid cells. The apparent K_m for the phosphorylation of 5-bromodeoxycytidine in extracts of mouse lymphoma cells was 2.0 mM, in contrast to a K_m of 10 μM for deoxycytidine phosphorylation. The apparent K_m for the phosphorylation of 5-methyldeoxycytidine by the mouse enzyme was 80 μM. The affinity of mouse deoxycytidine kinase for 5-iododeoxycytidine was lower than for 5-bromodeoxycytidine, whereas its affinity for 5-fluorodeoxycytidine was similar to that for deoxycytidine. With human deoxycytidine kinase the apparent K_m was 0.4 mM for 5-bromodeoxycytidine, as compared to 2.0 μM for deoxycytidine. These results suggest that the activity of deoxycytidine kinase is affected by the size of substitutions in position 5 of its substrate. This restricted substrate specificity of deoxycytidine kinase could limit the utilization of 5-bromodeoxycytidine for DNA synthesis.

INTRODUCTION

5-Bromo- or 5-iododeoxycytidine is incorporated into the DNA of murine cells as 5-bromo- or 5-iododeoxyuridylate (1, 2). This could occur by reactions catalyzed either by cytidine deaminase and thymidine kinase (5-bromodeoxycytidine → 5-bromodeoxyuridine → 5-bromodeoxyuridylate) or by deoxycytidine kinase and deoxycytidylate deaminase (5-bromodeoxycytidine → 5-bromodeoxycytidylate → 5-bromodeoxyuridylate).

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Cramer *et al.* (1) have reported that P815Y mouse neoplastic mast cells lacking thymidine kinase are resistant to the cytotoxic effects of 5-bromodeoxycytidine. We have found that inhibition of cytidine deaminase *in vivo* inhibits the incorporation of 5-bromodeoxycytidine into the DNA of mouse tissues (3). These results suggest that the utilization of 5-bromodeoxycytidine proceeds primarily by the cytidine deaminase–thymidine kinase pathway. Since 5-bromodeoxycytidylate has been shown to be a good substrate for deoxycytidylate deaminase (4), we have investigated the substrate specificity of deoxycytidine kinase in order to account for the comparative lack of activity of the deoxycytidine kinase–deoxycytidylate deaminase pathway.

METHODS

Chemicals. [5-^{3}H]Deoxycytidine and 5-bromo[6-^{3}H]deoxycytidine were purchased from New England Nuclear Corporation. Thymidine [γ-^{32}P]5′-triphosphate was purchased from International Chemical and Nuclear Corporation. Nucleoside 5′-triphosphates, deoxycytidine, 5-methyldeoxycytidine, 5-bromodeoxycytidine, and 5-iododeoxycytidine were purchased from Sigma Chemical Company. 5-Fluorodeoxycytidine was a generous gift from Dr. Jack J. Fox of the Sloan Kettering Institute for Cancer Research.

Growth of cells and preparation of extracts. P388 mouse ascites lymphoma cells were maintained by intraperitoneal transplantation in DBA/2 male mice. Cells were harvested 6 days after inoculation of 5×10^5 cells. The cells were washed twice with phosphate-buffered saline (0.8% sodium chloride, 0.02% potassium chloride, 0.15% sodium phosphate dibasic, and 0.02% potassium phosphate monobasic, pH 7.2). Erythrocytes were hemolyzed by suspending the cell pellet in 2 volumes of distilled water for 1 min, after which isotonicity was restored by addition of 1 volume of 2.7% sodium chloride and 5–10 volumes of phosphate-buffered saline. Cells were collected by centrifugation, and the procedure was repeated two or three times until hemolysis was complete. The cells were then suspended in 2–5 volumes of buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10% glycerol, 1.0 mM magnesium chloride, and 0.2 mM thymidine 5′-triphosphate. Cells were disrupted by sonication at 0°. The sonic extract was centrifuged at 105,000 $\times$ *g* at 4° for 90 min. The supernatant fluid was stored at −30°. Thymidine 5′-triphosphate or uridine 5′-triphosphate was required for enzyme stability, in the frozen state and at 37°.

RPMI 4098 cells, a normal human lymphoid cell line obtained from Associated Biomedic Systems, were grown in stationary culture in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) in an atmosphere of 5% carbon dioxide–95% air. Media, sera, and antibiotics were purchased from Grand Island Biological Corporation. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline, and an extract was prepared and stored as described for P388 cells.

Protein concentrations were determined by the method of Lowry *et al.* (5), with bovine serum albumin as standard.

Phosphorylation of ^{3}H-labeled nucleosides. The assay was a modification of that described by Kessel (6). The reaction mixture contained 5 mM magnesium chloride, 10 mM sodium fluoride, 10 mM potassium phosphate (pH 8.0), 50 mM Tris-HCl (pH 8.0), [5-^{3}H]deoxycytidine or 5-bromo[6-^{3}H]deoxycytidine, and either adenosine 5′-triphosphate, uridine 5′-triphosphate, or thymidine 5′-triphosphate as the phosphate donor in a total volume of 0.1 ml. After incubation at 37°, the reaction was terminated in boiling water, and 0.05-ml aliquots were applied to 2.4-cm-diameter discs of Whatman DE81 chromatography paper. The discs were washed four times with distilled water and twice with 95% ethanol. The discs were then dried, and radioactivity was determined by liquid scintillation spectrometry. Product formation was proportional to incubation time in all experiments for which reaction rates are reported. Initial reaction rates were proportional to the amount of extract added to the reaction mixture.

Phosphorylation of unlabeled nucleosides. In order to assay phosphorylation of unlabeled nucleosides, thymidine [γ-^{32}P]5′-triphosphate was used as the phosphate donor and the nucleoside monophosphate product was isolated by chromatography on DEAE-impregnated paper (Whatman DE81). Since good chromatographic separation of the nucleotide product from the donor triphosphate was essential, it was necessary to prevent the formation of deoxycytidine 5′-di- or -triphosphate. For this reason thymidine [γ-^{32}P]5′-triphosphate was used as the phosphate donor. Deoxycytidine kinase can utilize thymidine 5′-triphosphate as well as adenosine 5′-triphosphate as a phosphate donor (7, 8), but thymidine 5′-triphosphate cannot be used by deoxycytidylate kinase (9). Therefore the only product formed un-

der these conditions is deoxycytidine 5'-monophosphate (6).

Before use in this assay, the enzyme preparation was dialyzed for 18 hr against two changes of 1-liter aliquots of the buffer used for enzyme preparation, in order to remove endogenous nucleosides and nucleotides. Thymidine [γ-^{32}P]5'-triphosphate (0.2 mM, 20 μCi/μmole) was used as the phosphate donor in the standard reaction mixture. Controls consisted of reaction mixtures which did not contain acceptor nucleosides. After incubation at 37°, tubes were placed in ice, and the reaction was terminated by addition of 0.9 ml of phosphate precipitation reagent (10), consisting of 0.57 N perchloric acid, 2.9% ammonium molybdate, and 29 mM triethylamine hydrochloride (pH 5.0). Precipitated inorganic phosphate and protein were removed by centrifugation, and 10 μl of the supernatant fluid were chromatographed on Whatman DE81 chromatography paper with 0.1 M sodium formate (pH 3.4). The R_F values of deoxycytidine 5'-monophosphate and thymidine 5'-triphosphate were 0.4 and 0.1, respectively. The positions of radioactive peaks were determined with a Baird-Atomic radiochromatogram scanner. The radioactive peaks and corresponding areas of control chromatograms were cut out, and radioactivity was determined by liquid scintillation spectrometry.

Cytidine deaminase. The activity of cytidine deaminase was determined by a modification of the method of Durham and Ives (11) in reaction mixtures identical with the one described above. Following incubation at 37°, the reaction was terminated in boiling water. A 20-μl aliquot was applied to Whatman DE81 chromatography paper together with 10 μl each of deoxyuridine (10 mg/ml), deoxycytidine (10 mg/ml), and deoxycytidine 5'-monophosphate (20 mg/ml). After chromatography with 0.03 N formic acid, the standards were identified under ultraviolet light, the ultraviolet light-absorbing areas were cut out, and radioactivity was determined by liquid scintillation spectrometry. The R_F values of deoxycytidine, deoxyuridine, and deoxycytidine 5'-monophosphate were 0.6, 0.4, and 0.1, respectively.

RESULTS

5-Bromo[6-^{3}H]deoxycytidine, at concentrations of 20, 100, and 250 μM, was not phosphorylated at a significant rate, compared to the rate of [5-^{3}H]deoxycytidine phosphorylation, in extracts of P388 mouse lymphoma cells (Fig. 1). Deoxycytidine kinase from normal human lymphoid cells, like the murine enzyme, was unable to phosphorylate 20 μM 5-bromo[6-^{3}H]deoxycytidine, although 100 μM 5-bromo[6-^{3}H]deoxycytidine was phosphorylated at 18% of the rate of [5-^{3}H]deoxycytidine (Fig. 2). Comparable results were obtained with both the murine and human enzymes whether adenosine 5'-triphosphate or thymidine 5'-triphosphate was used as phosphate donor.

In order to demonstrate that ^{3}H-labeled nucleotide formation from [5-^{3}H]deoxycyti-

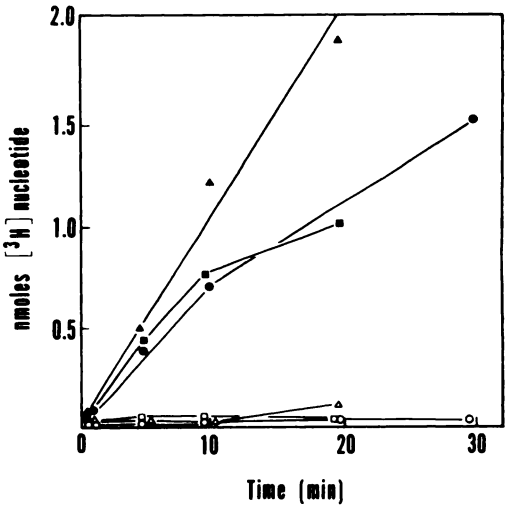

FIG. 1. *Phosphorylation of [5-^{3}H]deoxycytidine and 5-bromo[6-^{3}H]deoxycytidine by deoxycytidine kinase of P388 mouse lymphoma cells*

Reaction mixtures contained 5.0 mM adenosine 5'-triphosphate and 0.18 mg of 105,000 × *g* supernatant protein of P388 cells in a 0.1-ml volume. Nucleotide formation was determined by the DEAE disc method. [5-^{3}H]Deoxycytidine concentrations were 20 μM (●), 100 μM (■), and 250 μM (▲). 5-Bromo[6-^{3}H]deoxycytidine concentrations were 20 μM (○), 100 μM (□), and 250 μM (△). The specific activities of [5-^{3}H]deoxycytidine and 5-bromo[6-^{3}H]deoxycytidine were identical at each concentration: 20 μM, 25 μCi/μmole; 100 μM, 5 μCi/μmole; 250 μM, 2 μCi/μmole.

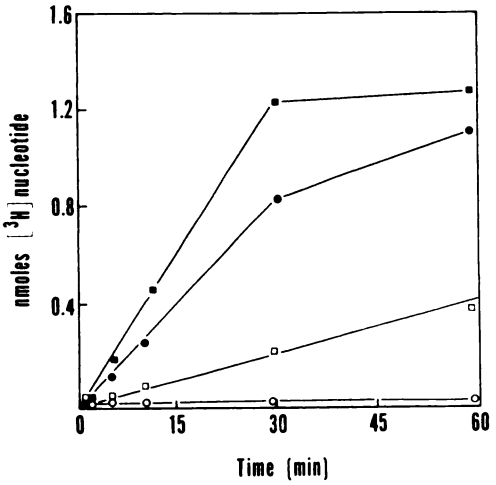

FIG. 2. *Phosphorylation of [5-³H]deoxycytidine and 5-bromo[6-³H]deoxycytidine by deoxycytidine kinase from RPMI 4098 human lymphoid cells*

Reaction mixtures contained 5.0 mM thymidine 5′-triphosphate and 0.15 mg of 105,000 × *g* supernatant protein of RPMI 4098 cells in a 0.1-ml volume. Nucleotide formation was determined by the DEAE disc method. [5-³H]Deoxycytidine concentrations were 20 μM (●) and 100 μM (■). 5-Bromo[6-³H]deoxycytidine concentrations were 20 μM (○) and 100 μM (□). The specific activities of [5-³H]deoxycytidine and 5-bromo[6-³H]deoxycytidine were identical at each substrate concentration: 20 μM, 25 μCi/μmole; 100 μM, 5 μCi/μmole.

dine was in fact catalyzed by deoxycytidine kinase, the effects of thymidine 5′-triphosphate and deoxycytidine 5′-triphosphate on the phosphorylation of [5-³H]deoxycytidine were studied. Nucleotide formation from [5-³H]deoxycytidine was increased by thymidine 5′-triphosphate and inhibited more than 98% by deoxycytidine 5′-triphosphate (Table 1). These results are similar to those obtained with purified preparations of deoxycytidine kinase, which is inhibited by deoxycytidine 5′-triphosphate (6, 7, 12). If the alternative mode of nucleotide formation, deamination of the nucleoside followed by phosphorylation by thymidine kinase, were being assayed, one would instead predict inhibition by thymidine 5′-triphosphate, which is a feedback inhibitor of thymidine kinase (13).

In order to exclude further the possibility that nucleoside deamination might provide

TABLE 1

Effects of thymidine 5′-triphosphate and deoxycytidine 5′-triphosphate on phosphorylation of [5-³H]deoxycytidine

Reaction mixtures contained 20 μM [5-³H] deoxycytidine (25 μCi/μmole), 5 mM adenosine 5′-triphosphate, and 0.15 mg of 105,000 × *g* supernatant protein of P388 cells in a 0.1-ml volume. Thymidine 5′-triphosphate and deoxycytidine 5′-triphosphate were added at concentrations of 0.5 mM. The formation of ³H-labeled nucleotides was determined by the DEAE disc method.

Added effector	Rate of [5-³H]deoxycytidine phosphorylation
	nmoles/hr
None	3.0
Thymidine 5′-triphosphate	4.8
Deoxycytidine 5′-triphosphate	<0.1

an alternative pathway for nucleotide formation, or interfere with determination of deoxycytidine kinase activity by deaminating significant amounts of substrate, the deamination and phosphorylation of deoxycytidine were assayed simultaneously by chromatographic analysis of the products formed in a reaction mixture containing 50 μM [5-³H]deoxycytidine and 0.2 mM thymidine 5′-triphosphate as substrates. No cytidine deaminase activity was detected in the P388 cell extracts (Table 2).

The phosphorylation of 5-substituted deoxycytidine analogues, in addition to 5-bromodeoxycytidine, was studied in order to investigate further the substrate specificity of mouse deoxycytidine kinase. Since these analogues were not available as radioactively labeled compounds, their phosphorylation was assayed utilizing thymidine [γ-³²P]5′-triphosphate as the phosphate donor, followed by chromatographic isolation, on DE81 chromatography paper eluted with 0.1 M sodium formate, of the ³²P-labeled nucleoside monophosphate product. With all nucleoside substrates studied, the ³²P-labeled nucleoside monophosphate product chromatographed with the same R_F (0.4) as deoxycytidine 5′-monophosphate. The enzyme preparation used in these experiments catalyzed the phosphorylation of deoxycytidine, 5-fluoro-

TABLE 2

Deoxycytidine kinase and deoxycytidine deaminase in extracts of P388 cells

Reaction mixtures contained 50 μM [5-^{3}H] deoxycytidine (10 μCi/μmole), 0.2 mM thymidine 5′-triphosphate, and 0.21 mg of P388 105,000 × *g* supernatant protein in a 0.1-ml volume. Aliquots (20 μl) of the reaction mixtures were chromatographed with carrier deoxyuridine, deoxycytidine, and deoxycytidine 5′-monophosphate on DE81 chromatography paper with 0.03 N formic acid. The ultraviolet light-absorbing areas corresponding to deoxyuridine (R_f 0.4), deoxycytidine (R_f 0.6), and deoxycytidine 5′-monophosphate (R_f 0.1) were cut out and counted in a liquid scintillation spectrometer.

Incubation time at 37°	Radioactivity recovered		
	Deoxycytidine	Deoxyuridine	Deoxycytidine 5′-monophosphate
min	*cpm*	*cpm*	*cpm*
0	1000	20	10
60	700	10	150

deoxycytidine, 5-methyldeoxycytidine, and 5-bromodeoxycytidine at a linear rate for 1–2 hr at 37°. To obtain the maximum sensitivity in kinetic experiments utilizing thymidine [γ-^{32}P]5′-triphosphate, reaction mixtures were incubated for 1 hr. The rate of deoxycytidine phosphorylation determined with this assay, in a reaction mixture containing 100 μM deoxycytidine, 0.2 mM thymidine [γ-^{32}P]5′-triphosphate, and 0.15 mg of P388 105,000 × *g* supernatant protein, was 1.5 nmoles/hr, which is comparable to a rate of 1.3 nmoles/hr determined in a parallel reaction mixture in which [5-^{3}H]deoxycytidine was used to assay deoxycytidine phosphorylation by the DEAE disc method.

The rate of phosphorylation of 5-fluorodeoxycytidine was approximately equal to that of deoxycytidine phosphorylation (Table 3). 5-Methyldeoxycytidine was phosphorylated at approximately 30% of the rate of deoxycytidine. Phosphorylation of 5-bromodeoxycytidine was detected at approximately 20% of the rate of deoxycytidine phosphorylation at a concentration of 0.5 mM, and at 32% of the rate of deoxycytidine phosphorylation at a concentration of 5.0 mM. No phosphorylation of 5-iododeoxycytidine was observed at a concentration of 0.5 mM, although 5.0 mM 5-iododeoxycytidine was phosphorylated at 15% of the rate of deoxycytidine.

TABLE 3

Phosphorylation of 5-substituted deoxycytidine analogues by deoxycytidine kinase of P388 cells

Reaction mixtures contained thymidine [γ-^{32}P] 5′-triphosphate (0.2 mM, 20 μCi/μmole) and 0.15 mg of P388 105,000 × *g* supernatant protein which had been dialyzed to remove endogenous nucleosides and nucleotides. The rate of nucleotide formation was determined by isolation of the nucleotide monophosphate product on Whatman DE81 chromatography paper after 1 hr of incubation at 37°.

Nucleoside substrate	Rate of Nucleotide Formation	
	0.5 mM substrate	5.0 mM substrate
	nmoles/hr	
Deoxycytidine	1.8	2.4
5-Fluorodeoxycytidine	1.6	1.8
5-Methyldeoxycytidine	0.6	0.8
5-Bromodeoxycytidine	0.4	0.8
5-Iododeoxycytidine	<0.1	0.4

Kinetic parameters for 5-fluorodeoxycytidine, 5-methyldeoxycytidine, and 5-bromodeoxycytidine were determined with these nucleosides as substrates for murine deoxycytidine kinase and as inhibitors of [5-^{3}H]deoxycytidine phosphorylation (Table 4). All three nucleosides were competitive inhibitors of deoxycytidine phosphorylation (Fig. 3), with K_i values of 3, 87, and 1600 μM, respectively. The K_m values for 5-methyldeoxycytidine and 5-bromodeoxycytidine, 71 and 2100 μM, respectively, are in good agreement with the K_i values determined for these nucleosides. The K_m for 5-fluorodeoxycytidine could not be determined because high nucleoside concentrations, in large excess over the K_i of 3 μM, are required to assay phosphorylation of unlabeled nucleosides. The maximal velocities for phosphorylation of deoxycytidine and 5-fluorodeoxycytidine were equal, whereas those for 5-methyldeoxycytidine and 5-bromodeoxy-

TABLE 4

Kinetic parameters for phosphorylation of 5-substituted deoxycytidine analogues by P388 deoxycytidine kinase

The K_m and V_{max} values for deoxycytidine and the K_i values for 5-fluorodeoxycytidine, 5-methyldeoxycytidine, and 5-bromodeoxycytidine were determined from Fig. 3. The V_{max} value for 5-fluorodeoxycytidine was estimated from Table 3. The K_m and V_{max} values for 5-methyldeoxycytidine and 5-bromodeoxycytidine were determined in reaction mixtures which contained 0.2 mM thymidine [γ-^{32}P]5′-triphosphate (20 μCi/μmole) and 0.15 mg of dialyzed P388 105,000 × *g* supernatant protein. The rate of nucleotide formation was determined as described in Table 3. The concentrations of 5-methyldeoxycytidine were 25, 100, 200, and 500 μM. The concentrations of 5-bromodeoxycytidine were 0.5, 1.0, 5.0, and 10.0 mM.

Nucleoside	K_m	K_i	V_{max}
	μM	μM	*nmoles/hr*
Deoxycytidine	11		1.7
5-Fluorodeoxycytidine	—[a]	3	1.8
5-Methyldeoxycytidine	71	87	0.7
5-Bromodeoxycytidine	2100	1600	1.1

[a] Not determined.

cytidine were 60% and 40% lower, respectively.

For comparison with the mouse enzyme, kinetic parameters for the phosphorylation of [5-^{3}H]deoxycytidine and 5-bromo[6-^{3}H]-deoxycytidine were determined with human deoxycytidine kinase in extracts of RPMI 4098 cells. Reaction rates were measured in mixtures which contained 5.0 mM thymidine 5′-triphosphate and 1.0, 2.5, 5.0, 10, or 20 μM [5-^{3}H]deoxycytidine or 20, 50, 100, or 250 μM 5-bromo[6-^{3}H]deoxycytidine. K_m and V_{max} values were determined from linear Lineweaver-Burk plots. The apparent K_m for [5-^{3}H]deoxycytidine was 2.0 μM, whereas the apparent K_m for 5-bromo[6-^{3}H]deoxycytidine was 400 μM. The V_{max} values, determined in reaction mixtures which contained 0.15 mg of protein, were 5.2 nmoles/hr for [5-^{3}H]deoxycytidine and 3.3 nmoles/hr for 5-bromo-[6-^{3}H]deoxycytidine.

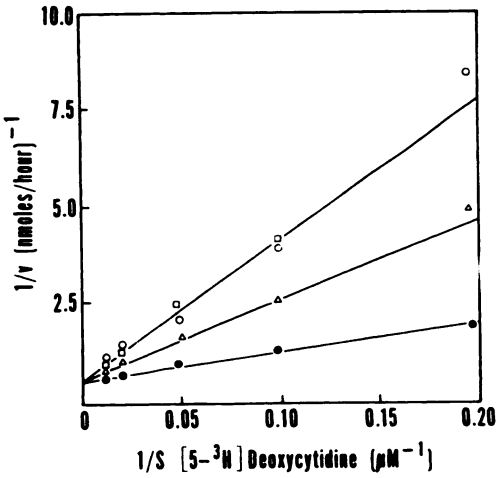

FIG. 3. *Inhibition of [5-^{3}H]deoxycytidine phosphorylation by 5-fluorodeoxycytidine, 5-methyldeoxycytidine, and 5-bromodeoxycytidine*

Reaction mixtures contained 0.2 mM thymidine 5′-triphosphate and 0.15 mg of 105,000 × *g* supernatant protein of P388 cells in a volume of 0.1 ml. The rate of nucleotide formation from [5-^{3}H] deoxycytidine was determined by the DEAE disc method after 30 min of incubation at 37°. Reaction mixtures contained various concentrations of [5-^{3}H]deoxycytidine and either no inhibitor (●), 7.5 μM 5-fluorodeoxycytidine (○), 200 μM 5-methyldeoxycytidine (△), or 5.0 mM 5-bromodeoxycytidine (□). The specific activities of [5-^{3}H]deoxycytidine were: 5 μM, 100 μCi/μmole; 10 μM, 50 μCi/μmole; 20 μM, 25 μCi/μmole; 50 μM, 10 μCi/μmole; 100 μM, 5 μCi/μmole.

DISCUSSION

The apparent K_m values for the phosphorylation of 5-substituted deoxycytidine analogues by mouse and human deoxycytidine kinase varied more than 200-fold, depending on the size of the 5-substitution. Since the V_{max} values of the deoxycytidine analogues varied less than 3-fold, it appears that 5-substituted deoxycytidine analogues differ in their affinity for binding to the enzyme. 5-Methyldeoxycytidine has a lower affinity for the enzyme than does deoxycytidine, and the affinity of deoxycytidine kinase for 5-halogenated deoxycytidine analogues is inversely related to the van der Waals radius of the halogen. The lower affinity of the enzyme for 5-bromodeoxycytidine compared to 5-methyldeoxycytidine could indicate an effect of the electronegativity of the halogen,

although such an effect is not apparent in the case of 5-fluorodeoxycytidine.

The restricted substrate specificity of deoxycytidine kinase can account for the lack of significant incorporation of 5-bromodeoxycytidine into DNA by the deoxycytidine kinase–deoxycytidylate deaminase pathway (1, 3). The finding that 5-fluorodeoxycytidine is a good substrate for deoxycytidine kinase leads to the prediction that its conversion to 5-fluorodeoxyuridylate could occur by the sequential actions of deoxycytidine kinase and deoxycytidylate deaminase. In contrast to their resistance to 5-bromodeoxycytidine, P815Y cells lacking thymidine kinase retain their sensitivity to 5-fluorodeoxycytidine (1), indicating that phosphorylation of the nucleoside by deoxycytidine kinase occurs to a significant extent in this cell line. Inhibition of the catabolism of 5-fluorodeoxycytidine by tetrahydrouridine increases the toxicity of 5-fluorodeoxycytidine in mice (3), suggesting that 5-fluorodeoxycytidine is phosphorylated to a significant extent *in vivo* by deoxycytidine kinase.

It has been observed that the deoxycytidine kinase induced in cells infected with herpes simplex virus catalyzes the phosphorylation of 5-bromodeoxycytidine as readily as deoxycytidine (14). This difference in substrate specificity between the cellular and virus-induced enzymes could be advantageous for therapy of herpes virus infections with 5-halogenated deoxycytidine analogues.

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