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STUDIES ON THE CYTOSTATIC ACTION, PHOSPHORYLATION AND DEAMINATION OF 5-AZACYTIDINE AND 5,6-DIHYDRO-5-AZACYTIDINE IN HELA CELLS

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Abstract—5-Azacytidine at 10 μM completely arrested the growth of HeLa cells, whereas 5,6-dihydro-5-azacytidine, a reduced stable analog of 5-azacytidine, at 100 and 200 μM did not inhibit their growth. 3,4,5,6-Tetrahydrouridine, a potent inhibitor of cytidine deaminase had no effect on cell growth at 100 μM, but in combination with 5,6-dihydro-5-azacytidine (100 μM) arrested cell growth completely. However, 3,4,5,6-tetrahydrouridine did not enhance the cytostatic action of 5-azacytidine at concentrations which were only slightly inhibitory to cell growth. Kinetic studies with HeLa cell preparations of cytidine kinase showed that 5-azacytidine is phosphorylated to a greater extent than 5,6-dihydro-5-azacytidine but neither analog is as good a substrate as cytidine. Both drugs are deaminated by cell free extracts but apparent K_m values indicate that the reduced 5-azacytidine compound has a 10-fold greater affinity for cytidine deaminase than the parent drug.

5-Azacytidine (azaC) has been shown to be an effective antitumor agent against L1210 leukemia and spontaneous tumors in mice [1, 2]. The drug has also been shown to have clinical effectiveness in the treatment of acute myelocytic leukemia [3, 4]. However, hydrolytic decomposition of azaC at neutral pH values can occur with opening of the triazine ring producing compounds of unknown biological effects [5]. 5,6-Dihydro-5-azacytidine hydrochloride (DHazaC), a reduced derivative of azaC, is not susceptible to hydrolytic attack [6]. DHazaC retains antitumor activity comparable to azaC in terms of extension of life span against L1210 leukemia in mice, although the optimal dose is 33-fold higher for the reduced analog [7]. Neil *et al.* [8] have shown that the oral activity of azaC in L1210 leukemic mice is increased by 3,4,5,6-tetrahydrouridine (THU), a potent inhibitor of cytidine deaminase, and it is thought that the presence of THU inhibits inactivation of azaC by blocking its deamination. However, none of the oral activity enhanced by THU can be related to the inhibition of cytidine deaminase in the tumor, since L1210 cells are void of enzymatic activity.

Unlike L1210 cells, HeLa cells have measurable amounts of cytidine deaminase activity and therefore, could be a good model for studying the direct effects of THU in combination with azaC and DHazaC. In this paper we examined the cytostatic action of azaC and DHazaC in the presence of THU and compared the phosphorylation and deamination of these cytidine analogs by cell free extracts.

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MATERIALS AND METHODS

Methods. 5-Azacytidine (azaC) was purchased from Monsanto Chemicals and (DHazaC) was synthesized by reduction of azaC with $NaBH_4$ as described by Beisler *et al.* [6]. THU (NSC–112907) was supplied by the Drug Development Branch, DCT, NCI and glutamate dehydrogenase was purchased from Boehringer Mannheim (1200 units/ml). 2[^{14}C]-Cytidine (40 μCi/μmole) was obtained from Schwarz/Mann and 4[^{14}C]azaC (45 μCi/μmole) from Monsanto Research Corp. 4[^{14}C]DHazaC was synthesized from labeled azaC using the $NaBH_4$ procedure and had a specific activity of 0.41 μCi/μmole.

Cell culture. HeLa cells were grown in Eagle's #2 medium supplemented with 10% fetal calf serum. Growth inhibition studies were carried out at 37° by incubating 5 ml volumes of cells (1.5×10^5 cells/flask) in tissue culture flasks (5 ml, 25 cm^2). Test compounds were dissolved in isotonic phosphate buffer at pH 7.4 and were sterilized by passage through Millipore Swinnex-13 filter units. One-tenth ml of stock solutions was added to exponentially growing cells. At 24 hr intervals two flasks of cells were washed with phosphate buffered saline followed by trypsinization at 37° for 10 min with Dulbecco's PBS containing 0.01% EDTA and 0.25% Trypsin. Cells were dispersed by aspiration and counted with a Model B Coulter Counter. During treatment with the drugs some cells became detached and were assumed to be non-viable.

Enzyme assays. Deamination of cytidine and cytidine analogs was measured by a coupled assay to glutamate dehydrogenase in 37° in 20 mM phosphate buffer at pH 7.2 [9]. Cytidine kinase activity was measured by the isotope method of Furlong [10]. Assays were conducted in 20 mM Tris–Cl buffer at

ph 7.7 at 37° containing ATP (5 mM), $MgCl_2$ (5 mM) and different concentrations of 2[^{14}C]cytidine (1.20 μCi/μmole), 4[^{14}C]azaC (4.0 μCi/μmole) or 4[^{14}C]-DHazaC (0.41 μCi/μmole). HeLa cells (30 per cent suspension in 0.02 M potassium phosphate buffer at pH 7.2) were homogenized at 4° and then centrifuged at 100,000 *g* for 1 hr. The cell free supernatant extract was used for the source of cytidine kinase and deaminase. Activity is defined as μmoles of product formed per hr per ml reaction mixture and was linear during 20 min incubations. Protein determinations according to the method of Lowry *et al.* [11] were used with bovine serum albumin (Armour Pharmaceutical Co.) as standard.

RESULTS

AzaC at 1 μM appears to have a slight inhibitory effect on the growth of HeLa cells whereas, at 10 μM cellular proliferation was inhibited completely (Fig. 1a). THU at 100 μM had no effect on the normal growth rate of the cells, and in combination with 1 μM azaC did not potentiate the action of the drug. DHazaC at 100 μM and 200 μM (Fig. 1b) did not change the logarithmic growth pattern, but when given with 100 μM THU, the cytostatic action was drastically enhanced at both concentrations of the reduced drug.

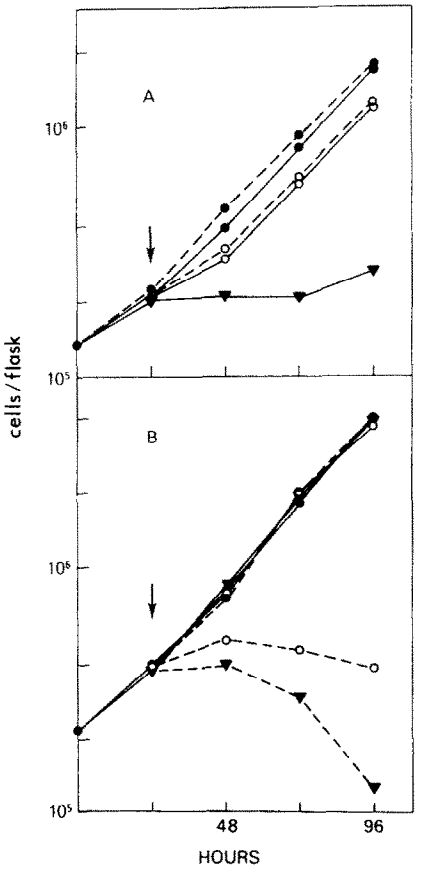

Fig. 1. Growth of HeLa cells in the presence of azaC, DHazaC and THU. (a) ●——●, control; ●----●, 100 μM THU; ○——○, 1 μM azaC; ○----○, 1 μM azaC plus 100 μM THU; ▼, 10 μM azaC. (b) ●——●, control; ●----●, 100 μM THU; ○——○, 100 μM DHazaC; ○----○, 100 μM DHazaC plus 100 μM THU; ▼——▼, 200 μM DHazaC; ▼----▼, 200 μM DHazaC plus 100 μM THU. Arrows indicate when drugs were administered.

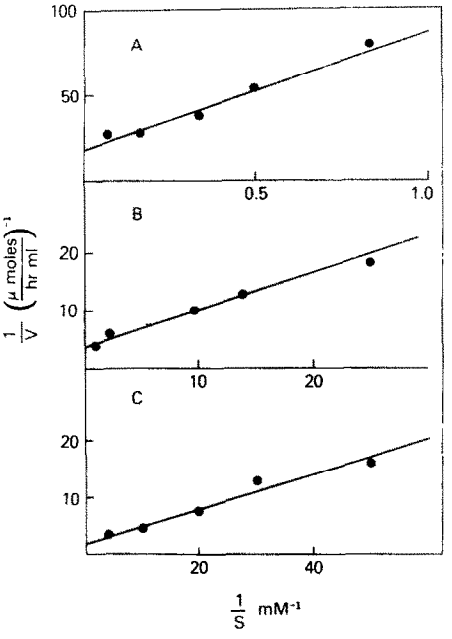

Fig. 2. Double reciprocal plots for cytidine kinase. See experimental section for details (a) DHazaC, (b) azaC and (c) cytidine.

Figure 2 illustrates that cytidine kinase from HeLa cells phosphorylates both azaC and DHazaC, but to a lesser extent than cytidine. At saturating substrate concentrations, azaC is phosphorylated at 55 per cent and DHazaC at 13 per cent the rate of the natural substrate. The apparent K_m values for azaC and DHazaC were approximately 2 and 19-fold higher than that of cytidine (0.12 mM), respectively (Table 1a). Figure 3 demonstrates that both azaC and DHazaC are substrates for cytidine deaminase, but at saturating concentrations the rate of deamination of cytidine is highest followed by azaC and DHazaC. However, the apparent K_m values (Table 1b) indicate that DHazaC binds to the enzyme more

Table 1. Kinetic parameters*

(A) Cytidine kinase		
Substrate	k_m (mM)	V_m (μmoles/ml/hr)
Cytidine	0.12	0.45
AzaC	0.22	0.25
DHazaC	4.2 (1.7)†	0.06
(B) Cytidine deaminase		
Substrate	k_m (μM)	V_m (μmoles/ml/hr)
Cytidine	14	0.55
AzaC	40	0.38
DHazaC	4	0.23

* The protein concentrations in the reaction mixtures were 50 μg/ml.

† DHazaC was found to be a competitive inhibitor using varying concentrations of labelled cytidine as substrate. The number in parenthesis is the apparent K_i value for DHazaC.

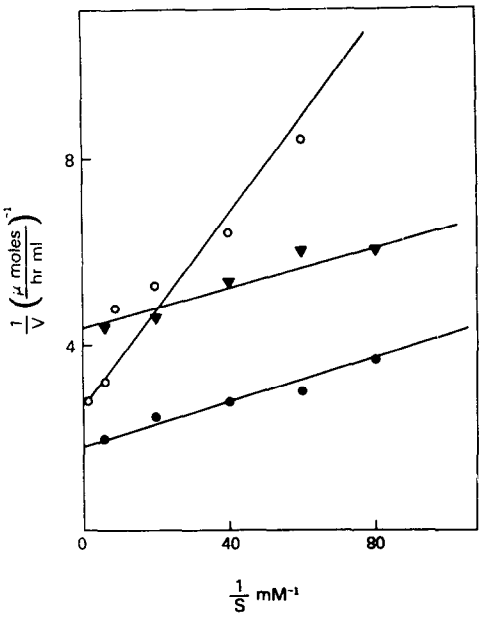

Fig. 3. Double reciprocal plots for cytidine deaminase. See experimental section for details. ●, cytidine; ○, azaC; ▼, DHazaC.

effectively than cytidine and 10-fold better than azaC. Regardless of the substrate, no deaminase activity was detected in the presence of 100 μM THU.

DISCUSSION

In a previous report from this laboratory DHazaC was found to be cytostatic toward L1210 cells grown *in vitro* but concentrations 10-fold higher than for azaC were necessary to achieve an equivalent response (10 μM vs 1 μM) [7]. However, with HeLa cells as shown here (Fig. 1) azaC (10 μM) inhibits growth completely whereas DHazaC, even at concentration of 200 μM, showed no effect on the growth rate. The addition of THU (100 μM) with azaC at 1 μM did not potentiate the action of the drug but in combination with DHazaC (100 and 200 μM) resulted in total inhibition of cell growth. As shown in Fig. 2 azaC and DHazaC are phosphorylated by HeLa cell extracts, but the kinetic constants (Table 1) show that binding to cytidine kinase and the rate of phosphorylation of DHazaC are considerably less than observed with cytidine or azaC. It has been suggested that azaC must be phosphorylated and presumably incorporated into nucleic acids in order to be an effective cytostatic agent [12, 13]. Since the kinetic parameters for cytidine kinase show DHazaC to be a much poorer substrate than azaC, this may be one of the reasons why the reduced analog is inactive in inhibiting the growth of HeLa cells except in the presence of THU (Fig. 1b).

Chabner *et al.* [14] have suggested that cytidine deaminase may in part be responsible for the inactivation of azaC and that THU could be a useful agent in prolonging the *in vivo* effectiveness of the drug. As shown in Fig. 1a, THU in combination with 1 μM azaC did not potentiate the action of azaC in HeLa cell cultures, but when the cells were exposed to the combination of inhibitor and DHazaC (100 μM, a concentration that had no effect on cell growth), growth was completely arrested after 24 hr (Fig. 1b). Kinetic studies with cytidine deaminase from HeLa cells indicate that DHazaC has a 10-fold greater affinity for the enzyme as compared to azaC and probably under physiological conditions would be deaminated more quickly than the parent drug. Therefore, the presence of THU in culture may be more effective in blocking the deamination or inactivation of DHazaC than azaC.

Since L1210 cells do not contain detectable amounts of cytidine deaminase activity, the enhancement of azaC inhibition of L1210 cells grown in mice by THU may be the result of blocking deamination in tissues other than the tumor cells [8]. The clinical usefulness of THU may depend on the ability of the inhibitor to block deamination in normal tissues as well as in tumor cells.

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