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DIDEOXYCYTIDINE PERMEATION AND SALVAGE BY MOUSE LEUKEMIA CELLS AND HUMAN ERYTHROCYTES

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Abstract—Transmembrane equilibration of dideoxycytidine (ddCyd) in P388 mouse leukemia cells and human erythrocytes was only 1% as rapid as that of uridine and 2′-deoxycytidine, which is mediated by the facilitated nucleoside transporter of these cells. ddCyd entry was nonsaturable up to a concentration of 1 mM but was partially inhibited by dipyridamole, nitrobenzylthioinosine and nucleosides, but not by nucleobases. Thus, entry was partly (70–80%) mediated, though very inefficiently, by the nucleoside carrier. Intracellular phosphorylation of ddCyd in P388 cells was also very inefficient compared to that of 2′-deoxycytidine and uridine and not rate limited by its slow entry into the cells.

2′,3′-Dideoxynucleosides are being investigated as potential antiviral agents [1–4], especially as inhibitors of the replication of the human immunodeficiency virus (HIV). To exert their antiviral effect, which is thought to be mediated at the level of viral DNA synthesis [1–4], dideoxynucleosides need to be salvaged by the cells. Nucleoside salvage in mammalian cells, in its simplest form, can be viewed as a two-component system comprised of carrier-mediated transport or non-mediated permeation across the plasma membrane followed by intracellular phosphorylation [5–7]. The facilitated, nonconcentrative nucleoside transporter present in all mammalian cells exhibits a broad substrate specificity, but the Michaelis–Menten constant differs greatly for different nucleosides ranging from 20 μM for 2-chloroadenosine to several millimolar for cytidine [5–7]. The first-order rate constants for transport (V_{max}/K_m), however, are similar for various natural nucleosides, which indicates that at physiological concentrations ($\leq$1 μM) they are transported with similar efficiency. Nucleoside kinases, on the other hand, exhibit much more restricted substrate specificities and, in general at least 10-fold greater substrate affinities than the nucleoside transporter [5].

2′,3′-Dideoxycytidine (ddCyd) and 2′,3′-dideoxyadenosine (ddAdo) have been shown to be taken up and phosphorylated by cultured cells [1–4, 8–11]. However, the efficiency of salvage seems to be low and to differ for various dideoxynucleosides, and it has not been ascertained to what extent dideoxynucleoside salvage is limited by entry into the cell or intracellular phosphorylation. One study [12] suggests that ddAdo entry into human T-cell lines is mainly non-mediated, since it is not inhibited by 20 μM nitrobenzylthioinosine (NBTI), a specific inhibitor of nucleoside transport [7]. In contrast, other studies [2, 13] indicate that ddCyd is a substrate for the facilitated nucleoside transporter of cultured human T lymphoblasts, but the efficiency and kinetics of transport and the non-mediated permeation of ddCyd were not analyzed. In the present study we compared the entry and the overall salvage of ddCyd in mouse leukemia cells with those of several natural nucleosides and examined permeation of ddCyd directly in human erythrocytes, which lack kinases for pyrimidine ribo- and deoxyribonucleosides.

MATERIALS AND METHODS

Cells. P388 and L1210 mouse leukemia cells were propagated in suspension culture as described previously [14, 15], harvested from late exponential phase cultures, and suspended in basal medium 42B (BM42B; Ref. 16) to 1–3 $\times$ 10^7 cells/ml. Erythrocytes from freshly drawn human blood were supplied by Dr J. Kersey (Department of Pathology, University of Minnesota), washed thrice in cold saline containing 5 mM Tris–HCl, pH 7.4 (Tris–saline), and suspended in the same to 4 $\times$ 10^8 to 1 $\times$ 10^9 cells/ml. Cells were enumerated with a Coulter counter.

Measurement of [^{3}H]*ddCyd uptake.* Suspensions of cells were supplemented with [^{3}H]ddCyd and potential inhibitors and competitors of its uptake as indicated in appropriate experiments. At appropriate times of incubation at 25°, the cells from 0.5-ml samples of suspension were collected by centrifugation through oil and analyzed for radioactivity as described previously [15, 17, 18]. Values for radioactivity in cell pellets were corrected for the amount of radioactivity trapped in the extracellular space, which was estimated by the use of [^{14}C]inulin [17] and converted to picomoles per microliter of cell water on the basis of the intracellular water space determined by the use of ^3H_2O [17] and the specific radioactivity of the substrate.

The acid-soluble pools of cells were extracted in several ways. In one procedure, cells labeled with [^{3}H]ddCyd were collected by centrifugation through oil directly into a layer of sucrose and 0.5 N trichloroacetic acid or 1 N perchloric acid, which rap-

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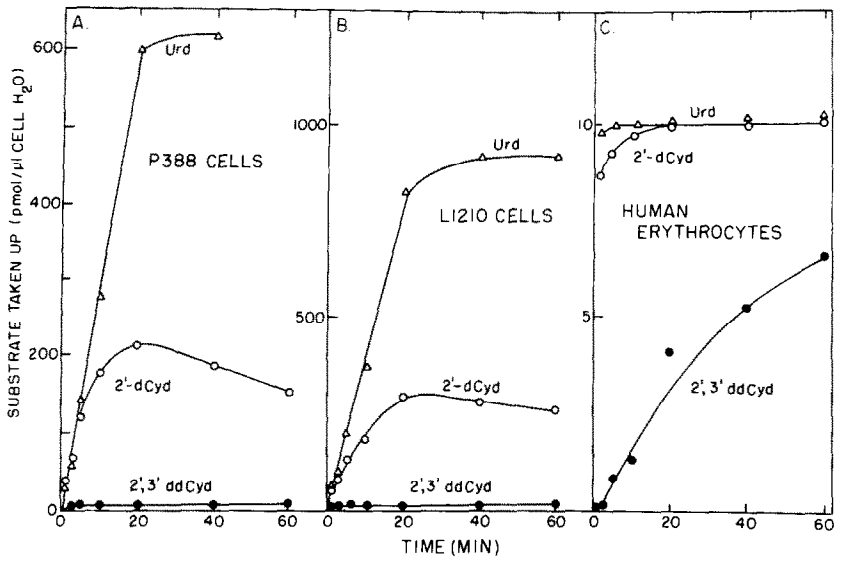

Fig. 1. Uptake of Urd, 2'-dCyd and 2',3'-ddCyd by P388 (A), and L1210 (B) mouse leukemia cells and by human erythrocytes (C). Samples of suspensions of 1.6×10^7 P388 or L1210 cells/ml of BM42B or 4.4×10^8 human red cells/ml of Tris–saline were supplemented with 10 µM [^{3}H]Urd (68 cpm/pmol), [^{3}H]dCyd (44 cpm/pmol) or [^{3}H]ddCyd (87 cpm/pmol). At the indicated times of incubation at 25°, the cells from duplicate 0.5-ml samples of each suspension were collected by centrifugation through oil and analyzed for radioactivity. All radioactivity values were corrected for substrate trapped in the extracellular space of cell pellets and converted to picomoles per microliter of cell water on the basis of an experimentally determined cell water space. All values are averages of duplicate samples.

idly quenches further metabolism [15]. Or the cells were collected by centrifugation through oil and the cell pellet was then extracted at 0° with trichloroacetic or perchloric acid or with 60% (v/v) ethanol at −20°. The acid extracts were further extracted with ether. Then the cell extracts as well as the culture fluid remaining above the oil layer were analyzed by ascending paper chromatography as described previously [15, 16] with solvent 28 (30 ml of 1 M ammonium acetate, pH 5.0, and 70 ml of 95% ethanol), which separates ddCyd, ddCMP, ddCDP and ddCTP or solvent 40 (39 ml butanol, 22 ml ethyl acetate, 22 ml ammonium hydroxide and 17 ml methanol) for the detection of potential degradation products of ddCyd. No degradation products were observed, and all cell extraction procedures yielded extracts with comparable composition.

Materials. [5-^{3}H]Uridine (Urd) and [5,6-^{3}H]ddCyd were purchased from Moravek Biochemicals (Brea, CA). They were >95% pure as assessed by chromatography with solvents 28 and 40. Unlabeled ddCyd and ddAdo were purchased from Pharmcia-P.L. Biochemicals (Piscataway, NJ). Other unlabeled nucleosides and nucleobases were obtained from the Sigma Chemical Co. (St Louis, MO) and NBTI from Calbiochem (San Diego, CA). Dipyridamole was a gift from Geigy Pharmaceuticals (Yonkers, NY) and pyrazofurin was supplied by the Division of Cancer Treatment, National Cancer Institute.

RESULTS AND DISCUSSION

Urd is generally rapidly salvaged by mammalian cells, including P388 and L1210 mouse leukemia cells [5, 19]. In the experiment depicted in Fig. 1A, the intracellular radioactivity from 10 µM [^{3}H]Urd exceeded that in the medium about 5-fold within 1 min of incubation at 25° and over 60-fold after about 20 min. In fact, net uptake of [^{3}H]Urd ceased at this time because the medium had become practically depleted of [^{3}H]Urd. dCyd salvage was also rapid but less efficient (Fig. 1, A and B), apparently because of the feedback regulation of dCyd kinase (see Ref. 20). Chromatographic analyses of the acid-soluble pools showed that >90% of the intracellular radioactivity derived from [^{3}H]Urd or [^{3}H]dCyd was associated with nucleotides (data not shown).

In contrast to the efficient salvage of Urd and dCyd, ddCyd accumulated in P388 and L1210 cells only very slowly (see Fig. 1 and Fig. 2B), the intracellular concentration of ^{3}H reached only about equilibrium with the extracellular concentration even after 60 min of incubation (Fig. 1, A and B, and see later experiments), and <10% of the intracellular ^{3}H was associated with nucleotides (data not shown). The latter result is in general agreement with those reported by Balzarini *et al.* [3]. In their study, L1210 cells had accumulated, depending on their growth stage, only between 144 and 700 nM ddCyd nucleotides after 4.5 hr of incubation with 1 µM [^{3}H]ddCyd at 37°.

We have shown previously [20] that preincubation of cultured cells with hydroxyurea, thymidine (dThd) or pyrazofurin enhances the salvage of dCyd and cytosine arabinoside (see Fig. 2A), presumably because these treatments result in altering the levels of dCTP and perhaps other pyrimidine nucleotides that regulate dCyd kinase. Balzarini *et al.* [3] reported that such treatments enhance the long-term formation of ddCyd nucleotides from [^{3}H]ddCyd in L1210 cells in low density cultures. We did not

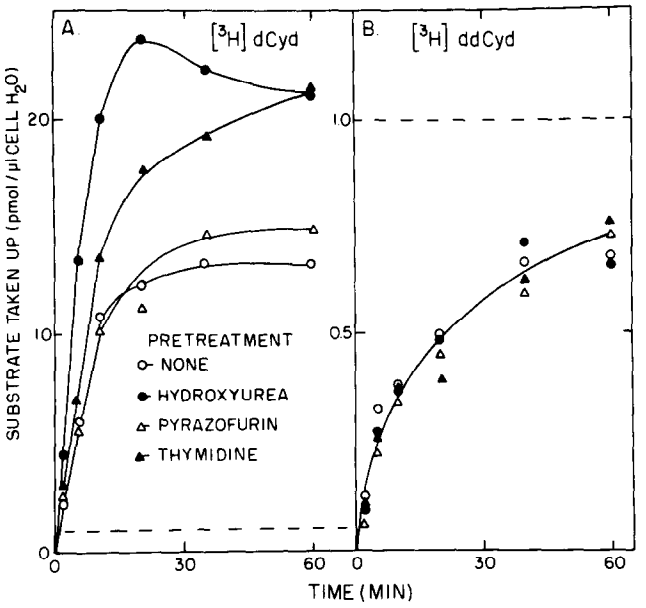

Fig. 2. Effect of pretreatment of P388 cells with hydroxyurea, thymidine or pyrazofurin on the salvage of dCyd (A) and ddCyd (B). Samples of a suspension of 1.2×10^7 cells/ml of BM42B were supplemented with 0.5 mM hydroxyurea, 100 μM dThd or 2 μM pyrazofurin and incubated at 37° for 2 hr. The suspensions were monitored for the uptake of 1 μM [^{3}H]dCyd (414 cpm/pmol) or [^{3}H]ddCyd (593 cpm/pmol) at 25° as described in the legend to Fig. 1. The broken lines indicate the intracellular concentration of ^{3}H equal to that in the medium.

Table 1. Intracellular concentrations of radiolabeled nucleotides in untreated and pretreated P388 cells after incubation with 1 μM [^{3}H]dCyd or [^{3}H]ddCyd*

	[^{3}H]dCyd			[^{3}H]ddCyd		
Pretreatment	dCTP	dCMP	dCyd	ddCTP	ddCMP	ddCyd
None	10	2.1	0.60	<0.1	<0.1	0.66
Hydroxyurea	18	3.5	0.42	<0.1	<0.1	0.65
Thymidine	18	3.6	0.50	<0.1	<0.1	0.75
Pyrazofurin	12	2.7	0.80	<0.1	<0.1	0.72

* The details of the experiment are described in the legend to Fig. 2. After 60 min of incubation at 25°, the acid-soluble pools were extracted from samples of cells and analyzed chromatographically with solvent 28. All values are in μM. These concentrations were calculated on the basis of the chromatographic separations and the total amounts of substrate accumulated by the cells (Fig. 2).

observe such an effect in higher density suspensions of P388 cells. The time-course of uptake of 1 μM [^{3}H]ddCyd was the same in untreated cells and cells preincubated with hydroxyurea, thymidine or pyrazofurin (Fig. 2B) and no significant phosphorylation of [^{3}H]ddCyd was observed (Table 1). In contrast, the hydroxyurea and thymidine-treated cells accumulated about 20 μM dCyd nucleotides when incubated under identical conditions with 1 μM [^{3}H]dCyd (Fig. 2A and Table 1).

The results indicate that the main rate-limiting step in the conversion of extracellular [^{3}H]ddCyd to intracellular nucleotides was its phosphorylation after entry into the cells. Entry into the cells, even though extremely slow compared to that of natural nucleosides transported by the nucleoside carrier (cf. Figs. 1A, B and 2B), did not limit significantly the salvage of ddCyd. The first-order rate constant for ddCyd entry into the cells, as estimated from the data in Fig. 2B, was about 0.016/min, which is only 1% of that (estimated as V_{max}/K_m for transport) observed for various natural nucleosides that are substrates for the nucleoside transporter of these cells [6, 7].

The slowness of permeation of ddCyd compared to the transport of the natural nucleosides is further illustrated by the data in Fig. 1C for human erythrocytes, in which pyrimidine nucleoside equilibration across the membrane can be measured uncomplicated by phosphorylation. The results show that 10 μM Urd and dCyd equilibrated with the intracellular space of these cells within 1–2 min, while the uptake of ddCyd was as slow as that in P388 and L1210 cells.

The following experiments examined the mechanism of permeation of ddCyd. The question arises

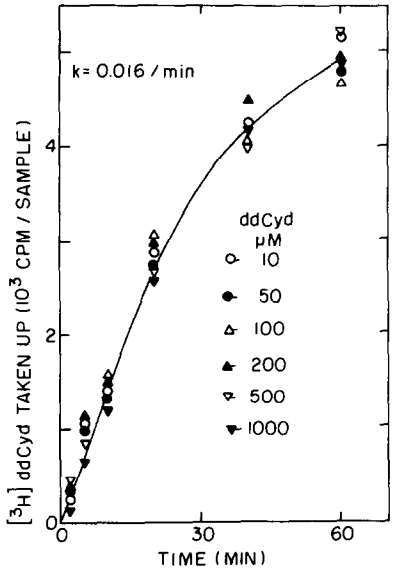

Fig. 3. ddCyd permeation into human erythrocytes as a function of the ddCyd concentration. Samples of a suspension of 7.5×10^8 cells/ml of Tris–saline were supplemented with the indicated concentrations of [^{3}H]ddCyd (370 cpm/μl; irrespective of concentration), and then uptake was measured at 25° as described in the legend to Fig. 1.

whether permeation is mainly non-mediated, because the first-order rate constant for its entry in relation to its lipid solubility was of the same magnitude as those reported for the non-mediated permeation of other nucleosides, nucleobases, and L-glucose [7]. The lipid solubility of ddCyd, as estimated by its solubility in octanol divided by its solubility in water (Z_{Oct}), was 0.048. The ratio of its first-order permeation rate constant to solubility (K/Z_{Oct}) was about 0.33/min. This latter value falls in the range reported for the non-mediated permeation of many substances with similar molecular weights, but differing in Z_{Oct} over a 600-fold range [7].

The results in Fig. 3 illustrate that the entry of ddCyd into human erythrocytes showed no sign of saturation up to a concentration of 1 mM. In the experiment, the concentration of [^{3}H]ddCyd was held constant while its specific activity was altered by addition of unlabeled ddCyd. It was not feasible to examine uptake at higher concentrations of ddCyd, because of its high cost. Nevertheless, the data indicate that, if ddCyd is a substrate for the nucleoside transporter, its transport K_m must be well above 1 mM.

Figure 4 illustrates that, in P388 cells and human erythrocytes, the permeation of 10 μM ddCyd was inhibited by 1 mM Urd, 1 mM formycin B, and 1 mM adenosine (Ado). Similar results were obtained for ddCyd uptake by L1210 cells (data not shown). Ado was more effective in inhibiting ddCyd entry than Urd or formycin B, which would be expected, if this inhibition is mediated at the level of the nucleoside transporter, since the K_m for Ado transport (about 100 μM) is considerably lower than those for Urd and formycin B transport (about 300 μM, Refs 7 and 21). In fact, the observed inhibitions of ddCyd uptake by 1 mM Urd, adenosine and formycin B approximated those anticipated on the basis of the known Michaelis–Menten constants for the transport of these nucleosides, competitive inhibition, an assumed K_m for ddCyd transport of 5 mM, and a 25% correction of the overall ddCyd entry rate for non-mediated permeation (see later Fig. 6). In contrast to the inhibition of ddCyd entry by other nucleosides, its uptake by human erythrocytes and P388 cells was little, if at all, affected by the nucleobases

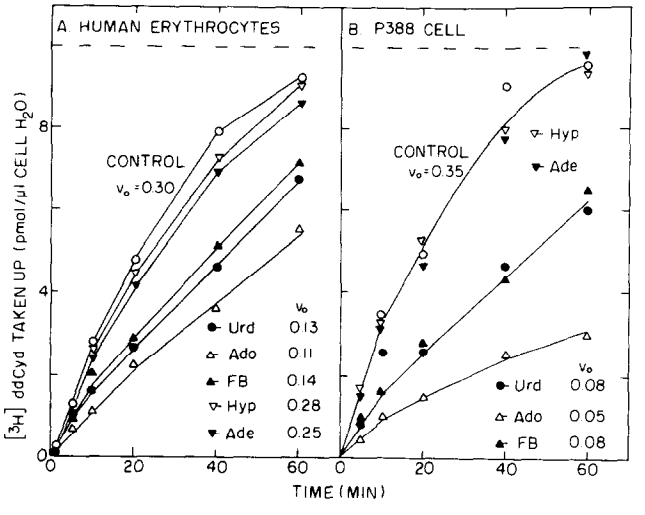

Fig. 4. Effects of other nucleosides and nucleobases on the uptake of [^{3}H]ddCyd by human erythrocytes (A) and P388 cells (B). The uptake of [^{3}H]ddCyd (10 μM) was measured under the same conditions as described in the legend to Fig. 1, except that, where indicated, the suspensions were supplemented with 1 mM unlabeled Urd, Ado, formycin B (FB), hypoxanthine (Hyp) or adenine (Ade) simultaneously with the [^{3}H]ddCyd. Initial velocities of uptake (v_o; in pmol/μl cell water·min) were estimated graphically. The broken lines indicate the intracellular concentration of substrate equal to that in the medium.

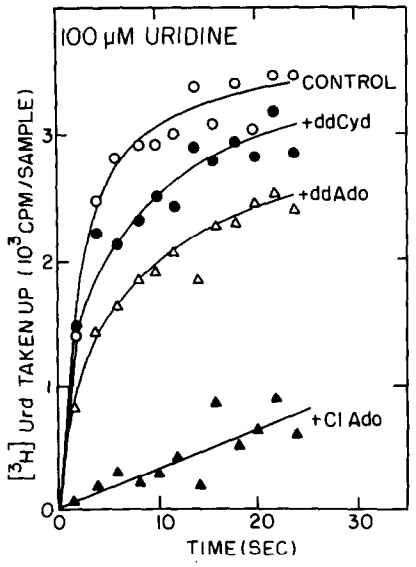

Fig. 5. Effects of ddCyd, ddAdo and 2-chloroAdo on the zero-*trans* influx of Urd in human erythrocytes at 25°. Transmembrane equilibration of 100 μM [^{3}H]Urd (3.2 cpm/pmol) was measured by rapid kinetic techniques by means of a dual syringe apparatus [17] in samples of a suspension of 1×10^9 human erythrocytes/ml of Tris–saline as described previously [7]. Where indicated, ddCyd, ddAdo or 2-chloroAdo was added to the cell suspensions to final concentrations of 1 mM simultaneously with the [^{3}H]Urd.

hypoxanthine and adenine (Fig. 4), which are transported by carriers distinct from the nucleoside transporter [7, 22]. Conversely, 1 mM ddCyd and ddAdo inhibited Urd transport in human erythrocytes, but only very inefficiently compared to the effects of other nucleoside transport substrates, such as 2-chloroadenosine (ClAdo; Fig. 5), which is efficiently transported with a K_m of about 20 μM [23, 24, and Plagemann, unpublished data].

ddCyd uptake in human erythrocytes (Fig. 6) and P388 cells (data not shown) was also strongly inhibited by NBTI and dipyridamole. Since NBTI is a transport inhibitor that is highly specific for the nucleoside carrier [7], the inhibition of ddCyd uptake must be mediated at the level of the nucleoside carrier. ddCyd entry into human erythrocytes was maximally inhibited about 75% by 0.5 to 2 μM NBTI (Fig. 6A). Since the nucleoside transporter of human erythrocytes exists entirely in the NBTI-sensitive form [7], the results indicate that nucleoside transport and non-mediated permeation account for about 75 and 25%, respectively, of the rate of entry of ddCyd. Maximum inhibition of ddCyd entry into P388 cells by NBTI was about 80%. Dipyridamole inhibited ddCyd entry into both human erythrocytes and P388 cells to a greater extent than NBTI (cf. Fig. 6, A and B), but this difference was not unexpected since dipyridamole, at high concentrations, also inhibits the permeation of substances, such as cytosine, which is thought to be non-mediated [7, 25]. Other experiments have shown that the efflux of 10 μM ddCyd from preloaded human erythrocytes was inhibited by the presence of 2 μM NBTI and 20 μM dipyridamole in the extracellular fluid to about the same extent as the influx of ddCyd in these cells (data not shown).

Overall, our results indicate that ddCyd permeation is partly non-mediated in human erythrocytes and P388 and L1210 mouse leukemia cells, but mostly mediated by the nucleoside carrier. This conclusion is consistent with the results of Ullman *et al.* [13] with a human T-lymphoblast line. However, ddCyd transport was about two orders of magnitude less efficient than that of various natural ribo- and deoxyribonucleosides, and the K_m of its transport fell well above 1 mM. The reason for the inefficient transport of ddCyd is not clear, since 2′-deoxyribonucleosides are transported about as efficiently as their ribonucleoside counterparts [6, 7] and 3′-dAdo also seems as efficiently transported as 2′-

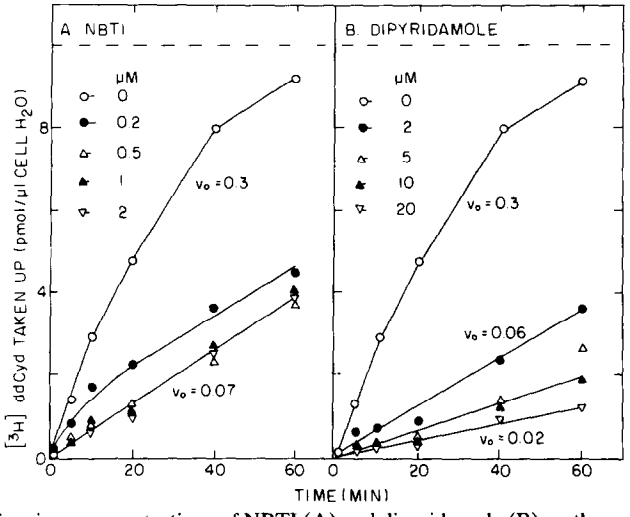

Fig. 6. Effects of various concentrations of NBTI (A) and dipyridamole (B) on the uptake of [^{3}H]ddCyd in human erythrocytes. Samples of a suspension of 7.5×10^8 human erythrocytes/ml of Tris–saline were supplemented with the indicated concentrations of NBTI and dipyridamole, and the uptake of 10 μM [^{3}H]ddCyd (26 cpm/pmol) was then monitored as described in the legend to Fig. 1. Initial uptake velocities (v_0; in pmol/μl water · *s*) were estimated graphically. The broken lines indicate the intracellular concentration of substrate equal to that in the medium.

dAdo and Ado [26]. However, preliminary experiments indicate that the transport of 2',3'-ddAdo is likewise very inefficient and entry is mainly non-mediated [12; Plagemann, unpublished data], and earlier studies have indicated that 2',3',5'-trideoxythymidine and 2',3',5'-trideoxyadenosine are relatively poor competitiors for Urd and dThd transport in Novikoff rat hepatoma cells [27]. Furthermore, 3'-azidothymidine and dideoxythymidine permeation into mammalian cells has been reported to be solely non-mediated [28, 29]. However, slow facilitated transport of 3'-azidothymidine may be difficult to detect, since 3'-azido-3'-deoxythymidine diffuses rapidly through membranes, because of its relatively high lipid solubility ($Z_{Oct} = 1.3$; Ref. 28). Thus, at least one OH-group at the 2' or 3' position of a nucleoside seems essential for its efficient transport by the nucleoside carrier. The polar OH group may be essential for the interaction of a nucleoside with the carrier, perhaps both during passage through the lipid bilayer and initial binding to the substrate binding site. On the other hand, the furanose ring of 2',3'-ddCyd, just as that of 3'-azido-3'-deoxythymidine, exhibits an unusual conformation [30], which may hinder its interaction with the carrier. To what extent these properties of 2',3'-deoxynucleosides affect their affinity for the carrier cannot be decided on the basis of our results with ddCyd. However, these affinities are strongly influenced by the nature of the nucleoside base; the K_m values range from 20–100 μM for various adenine nucleosides to several millimolar for cytosine nucleosides [6, 7].

The slow permeation of ddCyd, nevertheless, does not seem to limit significantly its uptake by the leukemia cells. It is the equally inefficient phosphorylation of ddCyd that is responsible for its poor salvage by the mouse leukemia cells and other mammalian cells [2, 8, 13]. Even stimulation of dCyd kinase activity by treatment of the P388 cells with thymidine, hydroxyurea or pyrazofurin enhanced ddCyd salvage only marginally when compared to the normal salvage of the natural nucleoside dCyd by the cells. The same properties of ddCyd discussed already have been considered as being responsible for its poor interaction with dCyd kinase [30]. This relatively poor salvage of ddCyd by cells may limit its potential as an antiviral agent.

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