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INHIBITION OF NUCLEOSIDE AND NUCLEOBASE TRANSPORT AND NITROBENZYLTHIOINOSINE BINDING BY DILAZEP AND HEXOBENDINE

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Abstract—The transport of 500 μM uridine by human erythrocytes and S49, P388 and L1210 mouse leukemia cells, Chinese hamster ovary (CHO) cells and Novikoff rat hepatoma cells was inhibited strongly by dilazep and hexobendine with similar concentration dependence, but the sensitivity of transport in the various cell types varied greatly; IC_{50} values ranged from 5–30 nM for human erythrocytes and S49 and P388 cells to >1 μM for CHO and Novikoff cells. The binding of nitrobenzylthioinosine (NBTI) to high-affinity sites on these cells ($K_d \approx 0.5$ nM) was inhibited by hexobendine and dilazep in a similar pattern. Furthermore, these drugs, just as dipyridamole and papaverine, inhibited the dissociation of NBTI from high-affinity binding sites but only at concentrations 10–100 times higher than those inhibiting uridine transport. In contrast, high uridine concentrations (>2 mM) accelerated the dissociation of NBTI. Dilazep also inhibited the transport of hypoxanthine, but only in those cell lines whose transporter is sensitive to inhibition by uridine and dipyridamole. Adenine transport was not inhibited significantly by dilazep in any of the cell lines tested, except for a slight inhibition in Novikoff cells. [^{14}C]Hexobendine equilibrated across the plasma membrane in human erythrocytes within 2 sec of incubation at 25°, but accumulated to 6–10 times the extracellular concentration in cells of the various cultured lines. Uptake was not affected by high concentrations of uridine, NBTI or dipyridamole. Hexobendine inhibited the growth of various cell lines to a lesser extent ($IC_{50} = \gtrsim 100$ μM) than dipyridamole ($IC_{50} = 15$–40 μM). At 40 μM, however, it completely inhibited the growth of S49 cells that had been made nucleoside dependent by treatment with methotrexate or pyrazofurin.

Mammalian cells possess a non-concentrative nucleoside transporter that transports all natural ribo- and deoxyribonucleosides, albeit with different efficiencies (for reviews see Refs. 1–3). Nucleoside transport is inhibited by a variety of substances, prime among them 6([4-nitrobenzyl]thio)-9-β-D-ribofuranosyl purine (nitrobenzylthioinosine or NBTI) and dipyridamole [1–3]. The inhibition of transport by NBTI correlates with its binding to high-affinity sites on the transporter ($K_d \sim 0.5$ nM; [3–6]), but most mammalian cells have been found to exhibit both NBTI-sensitive and NBTI-resistant nucleoside transport ([5–7]; see Table 1). No similar resistance of nucleoside transport to dipyridamole has been observed [5]. The mechanism of inhibition by dipyridamole and other lipophilic substances, which are structurally unrelated to nucleosides, is not understood, but seems to differ from that exerted by NBTI [8–10].

Nucleoside transport inhibitors are of practical interest, since some of them are potent coronary vasolidators. They may also be useful in enhancing the effectiveness of certain drugs in cancer chemotherapy [2] as well as in the selection of cell variants with amplified nucleoside transport genes by limiting nucleoside influx into cells that have been rendered nucleoside dependent by treatment with methotrexate [11], pyrazofurin [12, 13] or *N*-(phosphonacetyl)-L-asparate (PALA) [13]. The transport inhibition by dipyridamole has been well characterized [1], but this inhibitor may be of limited use in the latter applications, because it is toxic to cells at effective concentrations (see later). Other coronary vasolidators, such as lidoflazine, hexobendine and dilazep, have been reported to inhibit the salvage of adenosine by mammalian cells, presumably by inhibiting the transport step [3, 14–16], and have been found to compete with NBTI in equilibrium binding to cells [2, 3, 10, 17]. However, their direct effects on nucleoside transport and cell growth have not been analyzed in detail. In the present study, therefore, we have determined the effects of dilazep and hexobendine (see Fig. 1 for structures) on nucleoside transport, NBTI binding to and its dissociation from the transporter, and on cell growth in a variety of cell lines and, where applicable, in human erythrocytes.

MATERIALS AND METHODS

Cell cultures. Mouse L1210, P388 and L929 cells and Chinese hamster ovary (CHO) cells were propagated in suspension culture in Eagle's Minimal Essential Medium for suspension cultures supplemented with non-essential amino acids, sera and extra D-glucose as described previously [18]. Wild-type Novikoff rat hepatoma cells (N1S1-67) and a

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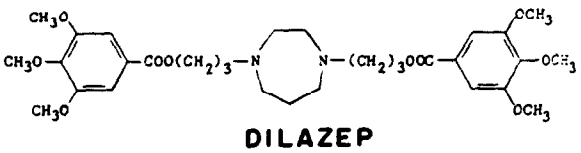
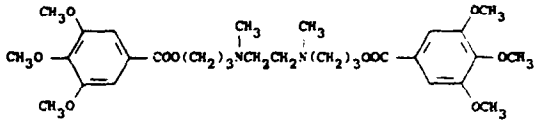


DILAZEP



HEXOBENDINE

Fig. 1. Structures of dilazep and hexobendine.

uridine kinase-deficient variant thereof (1-14-7) were propagated in the same manner, except in Swim's medium 67 [19]. S49 cells were supplied by Dr. B. Ullman and propagated in Eagle's Minimal Essential Medium supplemented with 5% (v/v) fetal bovine serum, non-essential amino acids and 10 mM additional D-glucose. Stock cultures were maintained in stationary suspension cultures in 75 and 150 cm^2 Corning tissue culture flasks and subcultured at 2-day intervals by a 1:5 to 1:8 dilution with fresh growth medium. Two-liter Schott roller bottles were inoculated with 100–200 ml of suspension harvested from stock cultures and incubated on a roller machine at about 0.3 rpm. The cultures were expanded to 1.0 to 1.5 liters by daily addition of more growth medium.

For measuring the inhibition of cell growth by various substances, cells from the various lines were seeded into 24-well tissue culture plates (about 1×10^5 cells/well) and incubated in a CO_2 incubator at 37° for 3 days. The cells were then enumerated in a Coulter counter. The cell lines were examined periodically for mycoplasma contamination by the uridine/uracil incorporation method [20]. No contamination was detected.

Human erythrocytes. Erythrocytes were a biproduct of lymphocyte isolations from freshly drawn blood and were supplied by Dr. J. Kersey (Department of Pathology). The cells were washed thrice in saline containing 5 mM Tris–HCl (pH 7.4; Tris–saline).

Measurement of uridine transport. Cells were harvested from mid to late exponential phase cultures and suspended to 2–5 $\times 10^7$ cells/ml of basal medium 42B (BM42B). The zero-*trans** influx of 500 μM uridine was measured in cell suspensions at 25° as described previously [5, 22]. Time courses of transmembrane equilibration of substrate were determined by rapid kinetic techniques (15 time points per time course). Data were evaluated by fitting an integrated rate equation, based on the simple carrier model, with directional symmetry and equal mobility of empty and substrate-loaded carrier with the Michaelis–Menten constant fixed at 250 μM [1]. The slopes of the progress curves at $t = 0$ were taken as initial velocities (v_{12}^{zt}). The equilibrium exchange* and zero-*trans* influx of 500 μM uridine in suspensions of about 5×10^8 human red blood cells/ml of Tris–saline were measured by rapid kinetic techniques as described previously [23].

Measurement of [^{14}C]*hexobendine uptake.* The uptake of [^{14}C]hexobendine was measured by rapid kinetic techniques as described for uridine transport. For the purpose of assessing any metabolic conversions of hexobendine, cells incubated with [^{14}C]hexobendine were centrifuged through an oil layer directly into a solution composed of 10% (w/v) sucrose and 0.5 N trichloroacetic acid, or the cells were collected by centrifugation and extracted with 1 mM EDTA at 100° or with methanol. The cell extracts and samples of the culture fluid were analyzed by ascending paper chromatography with solvents composed of 86:14 *n*-butanol:H_2O [13] or 40:40:25:8:13 *n*-butanol:2-butanone:acetone:NH_4OH:H_2O [24].

Equilibrium binding and dissociation of NBTI. Equilibrium binding was measured as described previously [5, 8]. Cell suspensions were mixed with [^{3}H]NBTI as indicated in appropriate experiments, and triplicate samples were analyzed for radioactivity (= bound + free NBTI). After about 30 min of incubation at 25°, other duplicate samples of suspension were centrifugally cleared and the supernatant fraction was also analyzed for radioactivity (= free NBTI). For measuring NBTI dissociation from cells, samples of a suspension equilibrated with 2 nM [^{3}H]NBTI were diluted 10-fold with cold balanced salt solution, and the cells were collected by centrifugation. The cells were suspended to one-half the original cell density in balanced salt solution containing 2 μM unlabeled NBTI and various other substances as indicated in appropriate experiments. At various times of incubation at 25°, duplicate samples were cleared of cells by centrifugation and the supernatant fraction was analyzed for radioactivity.

Materials. [5-^{3}H]Uridine and [^{3}H]NBTI were purchased from Moravek Biochemicals, Brea, CA. Unlabeled nucleosides were obtained from the Sigma Chemical Co., St. Louis, MO, and unlabeled NBTI from Calbiochem, San Diego, CA. Dipyridamole (Persantin) was a gift from Geigy Pharmachemicals, Yonkers, NY. Dilazep was a gift from Asta Werke AG, Frankfurt, Germany, and unlabeled and ^{14}C-labeled hexobendine were gifts from Chemie Linz, Linz, Austria. The [^{14}C]hexobendine had a specific radioactivity of 16 cpm/pmole, exhibited an absorption profile identical to that of the unlabeled compound (max = 265 nm; molar absorption coefficient $\approx 17 \times 10^3$) and was at least 90% pure as indicated by paper chromatography in three solvent systems. The octanol/water partition coefficient of [^{14}C]hexobendine was determined to be 8.0 by a procedure described previously [25].

* As defined by Stein [21] "zero-*trans*" designates the transport of a substrate from one side of the membrane to the other side, where its concentration is initially zero. "Equilibrium exchange" designates the unidirectional flux of radioactively labeled substrate from one side to the other side of the membrane, where substrate is held at equal concentration.

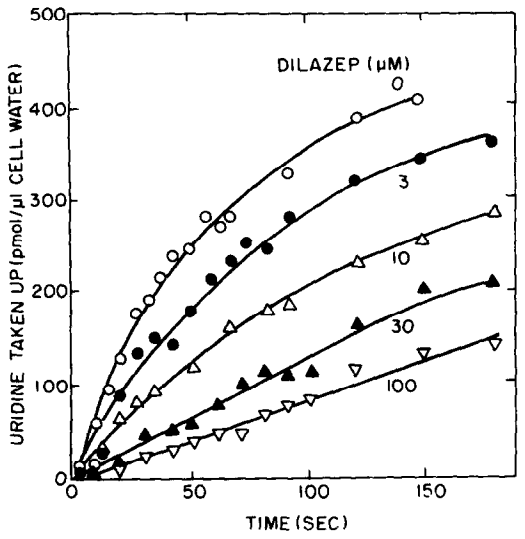


Fig. 2. Effect of dilazep on the zero-*trans* influx of uridine in Novikoff rat hepatoma cells. Samples of a suspension of 5×10^6 cells/ml of BM42B were supplemented with the indicated concentrations of dilazep, and then the transmembrane equilibration of 500 μM [^{3}H]uridine (1.4 cpm/pmole) was measured at 25° and the initial velocities of zero-*trans* entry (v_{12}^{zt}) were computed by integrated rate analysis as described under Materials and Methods. For the control cells, v_{12}^{zt} was 10.0 ± 0.5 pmoles/μl cell water·sec. The v_{12}^{zt} values for the inhibitor-treated cells are summarized in Fig. 3A as percent of control.

RESULTS AND DISCUSSION

Figure 2 shows representative time courses of the uptake of 500 μM [^{3}H]uridine by Novikoff rat hepatoma cells in the presence of the indicated concentrations of dilazep and illustrates the general procedure of measuring zero-*trans* influx of uridine in all experiments presented. We have documented already for several cell lines that, at these high uridine concentrations, little of the uridine accumulating in the cells during the first few minutes of incubation is phosphorylated or phosphorylyzed, so that uptake during this time period effectively reflects transmembrane equilibration of unmodified substrate [1, 5, 26]. In these and other experiments of this type initial zero-*trans* entry velocities (v_{12}^{zt}) were estimated as the zero time slopes of the progress curves [1].

The v_{12}^{zt} values for uridine transport in a number of cell lines as well as in human erythrocytes are plotted in Fig. 3 as a function of the concentrations of dilazep (frame A) and hexobendine (frame B). In both instances, the inhibition of uridine influx was dependent on inhibitor concentration, and it was similar for dilazep and hexobendine, but the sensitivity of uridine transport in the various cell types to the two substances differed greatly. The IC_{50} values ranged from 5–30 nM for human erythrocytes, S49 cells and P388 cells to 3–5 μM for Novikoff cells (Table 1). For all cell lines, the results were confirmed by a second experiment but with fewer inhibitor concentrations (data not shown). Comparable IC_{50} values have been reported for the inhibition of the uptake of 15 μM adenosine by dilazep in S49 and L5178Y cells (8 and 40 nM, respectively; [16]). The data for human red cells in Fig. 3 pertain to an equilibrium exchange of uridine, but similar IC_{50} values were obtained in another experiment for dilazep inhibition of both equilibrium exchange (7 nM) and zero-*trans* influx (10 nM) of uridine, which were measured in the same cell population. The differences in sensitivities of uridine transport in the various cell types to dilazep and hexobendine followed a pattern similar to that observed for dipyridamole but were greater than for dipyridamole (Table 1). Also, uridine transport in CHO cells was relatively resistant to inhibition by dilazep and hexobendine, whereas it is about as sensitive to dipyridamole as the uridine transport in L1210 and P388 cells. In contrast, NBTI-sensitive uridine transport of all types of cells exhibits about the same sensitivity to inhibition by NBTI, a finding that is

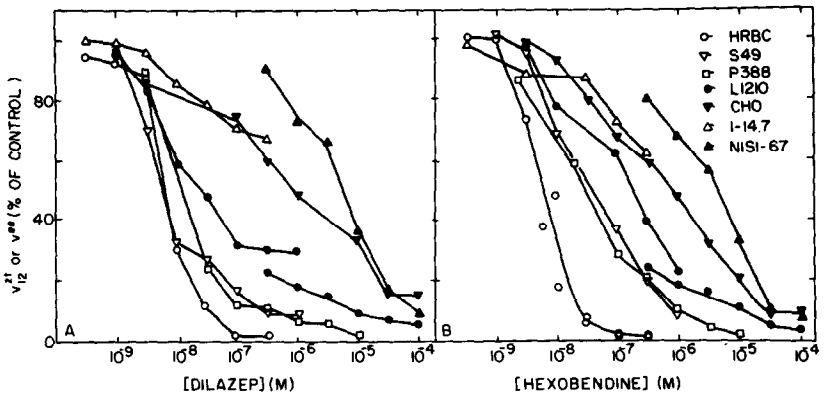


Fig. 3. Zero-*trans* influx of uridine in various cells as a function of concentration of dilazep (A) and hexobendine (B). The values for Novikoff cells were calculated from the data in Fig. 2. Experiments with other cell lines were conducted in the same manner as illustrated in Fig. 2. The v_{12}^{zt} values for untreated controls ranged from 10 to 20 pmoles/μl cell water·sec. The values for human erythrocytes (HRBC) are for an equilibrium exchange experiment with v^{ee} = 45 ± 5 pmoles/μl cell water·sec for the control cells.

Table 1. Comparison of the effects of dilazep, hexobendine, dipyridamole and NBTI on uridine transport in various types of cells

Cells	IC_{50} (nM) of uridine transport				NBTI-sensitive Urd transport* (% of total)
	Dilazep	Hexobendine	Dipyridamole*	NBTI*	
HRBC	~5	~5	~20	~6	~100
S49	~10	~30	~400	~1	~100
P388	~10	~30	~100	3–6	80–90
L1210	~30	~100	~300	3–6	80–90
CHO	~1,000	~1,000	~200	3–6	60–70
N1S1-67	~5,000	~3,000	~1,000	~10,000	<10

* Data are from Ref. 5, except for the inhibition of uridine transport by dipyridamole in S49 cells, and by NBTI in human red blood cells (HBRC), which had not been quantitated previously.

consistent with comparable kinetic parameters for NBTI binding to high-affinity sites on these cells [5, 6].

The sensitivities of various cell types to inhibition of equilibrium binding of NBTI to high-affinity sites by dilazep and hexobendine followed a pattern similar to that for the inhibition of uridine transport, but with some notable exceptions (cf. Table 1 and Fig. 4). The IC_{50} values for dilazep and hexobendine inhibition of NBTI binding were lowest for human erythrocytes and S49 cells (the residual resistant fraction of NBTI binding of about 20% in Fig. 4 represented non-specific binding, since it was also observed in the presence of 1 μM unlabeled NBTI; data not shown). The inhibition of NBTI binding by dilazep and hexobendine to human red blood cells was quantitatively similar to that reported previously (K_i = 0.3 and 1.9 nM, respectively; [17]), and an inhibition of NBTI binding by dilazep comparable to that in S49 cells has been reported for Nil 8 hamster cells (K_i = 15–32 nM; [10]). The IC_{50} values for inhibition of NBTI binding to red cells and S49 cells were comparable to those for the inhibition of uridine transport in these cells. On the other hand, the IC_{50} value for inhibition of NBTI binding in P388 cells was much higher than that for inhibition of uridine transport in these cells, whereas the opposite was the case for CHO cells. These discrepancies support the view that NBTI and other inhibitors that are structurally unrelated to nucleosides, such as dipyridamole, papaverine, dilazep and hexobendine, interact with the nucleoside transporter in different ways [8–10]. This conclusion is also indicated by an anomalously low inhibition of NBTI binding by high concentrations of dipyridamole [6] and the finding that high concentrations of dipyridamole and papaverine inhibit the dissociation of NBTI from high-affinity binding sites of human erythrocytes and hamster cells, whereas high concentrations of nucleosides accelerate dissociation ([8–10]; see Fig. 5).

Hexobendine and dilazep also strongly inhibited the dissociation of NBTI from cells in a concentration-dependent manner (Figs. 5 and 6). The IC_{50} value for the inhibition of NBTI dissociation from human erythrocytes was about 1 μM and about 10 times higher for its dissociation from CHO and P388 cells. These values compare to an IC_{50} of 2.5 μM for the inhibition of NBTI dissociation from human

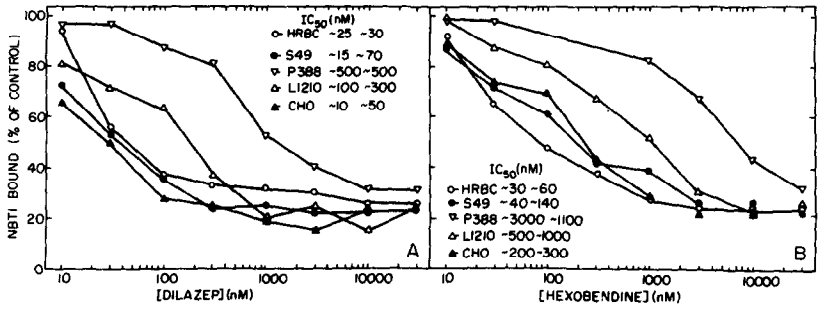


Fig. 4. Inhibition of equilibrium binding of NBTI by dilazep and hexobendine. Samples of cell suspensions (final densities = 5×10^6 to 4×10^7 cultured cells/ml and about 4×10^8 human red blood cells [HRBC]/ml) were equilibrated at 25° with 0.8 nM [^{3}H]NBTI and the indicated concentrations of dilazep or hexobendine or 3 μM unlabeled NBTI to assess non-saturable binding, and then they were freed of cells by centrifugation and the supernatant fraction was analyzed for radioactivity. All values are expressed as percent of the radioactivity bound by the cells in the absence of inhibitor (total binding), which in all cases represented 70–85% of the total [^{3}H]NBTI added. Non-saturable binding represented about 20% of the total bound. IC_{50} = inhibitor concentration inhibiting 50% of NBTI binding to high-affinity sites (total − non-saturable binding). The values listed in the first row are estimated from the dose–response curves presented; those in the second row are for another experiment conducted in the same manner.

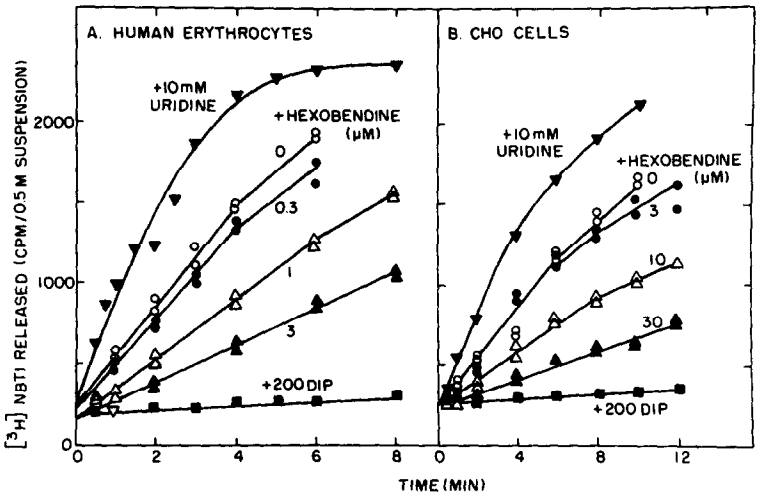


Fig. 5. Effects of hexobendine, dipyridamole (DIP), and uridine on the dissociation of NBTI from human erythrocytes (A) and CHO cells (B). Suspensions of 7×10^8 human red blood cells (HRBC)/ml or 4×10^7 CHO cells/ml were equilibrated with 2 nM [^{3}H]NBTI at 25° for 30 min. The cells were then collected by centrifugation and resuspended in medium containing 2 μM unlabeled NBTI and the indicated concentrations of hexobendine, DIP, or uridine as described under Materials and Methods. At various times of incubation at 25°, samples of each suspension were centrifuged, and the supernatant fractions were assayed for radioactivity. All values are averages of duplicate samples.

red cells by dipyridamole (data not shown). These values are about 100 times higher than the concentrations of inhibitor causing a 50% inhibition of NBTI binding and uridine transport in the same cells.

Similarly, an acceleration of NBTI-dissociation by uridine from erythrocytes was observed only at concentrations >2 mM (data not shown). Such concentrations are far in excess of those saturating the uridine binding site of the transporter [1] so that the acceleration is probably not explained simply as an interaction between the binding of uridine at the substrate binding site of the carrier, and the release of NBTI from some allosteric site. An analogous case can be made for the effects of the various transport inhibitors on NBTI dissociation. If they competed with NBTI for the same site on the transporter, they should not affect the rate of dissociation of bound radiolabeled-NBTI from the cells.

Table 2 demonstrates that dilazep inhibited hypoxanthine transport in NISI-67 and CHO cells (IC_{50} = 2–10 μM) to a similar extent as uridine transport (cf. Table 1), whereas it had only little effect on

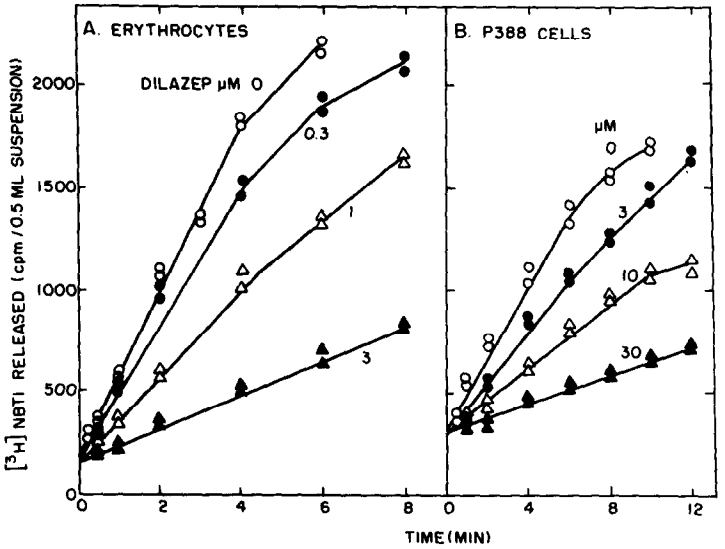


Fig. 6. Inhibition of the dissociation of [^{3}H]NBTI from human erythrocytes (A) and P388 cells (B) by dilazep. The experiments were conducted as described in the legend to Fig. 5. Cells equilibrated with 2 nM [^{3}H]NBTI were suspended in medium containing 2 μM unlabeled NBTI plus the indicated concentrations of dilazep, and the extracellular fluid was analyzed for radioactivity.

Table 2. Effects of dilazep on hypoxanthine (Hyp) and adenine (Ade) transport*

Substrate	Cell line	v_{12}^{ft} (pmoles/μl cells·sec) Control	+10 μM Dilazep	+100 μM Dilazep
Hyp	N1S1-67	28.4 ± 4.0	7.6 ± 0.4	1.7 ± 0.09 (94)†
	CHO	4.3 ± 0.2	1.9 ± 0.2	0.64 ± 0.08 (85)
	L1210	27.0 ± 3.5	25.7 ± 2.9	19.7 ± 1.5 (27)
	P388	23.4 ± 2.5	16.2 ± 1.7	13.4 ± 0.9 (33)
	HRBC	11.2 ± 0.4	8.9 ± 0.4	6.2 ± 0.4 (34)
Ade	N1S1-67	31.8 ± 9.7	19.2 ± 3.4	16.2 ± 3.1 (49)
	CHO	6.8 ± 0.7	5.9 ± 0.6	5.1 ± 0.6 (25)
	L1210	25.7 ± 5.3	25.4 ± 5.1	28.8 ± 4.7 (0)
	P388	21.3 ± 5.2	19.3 ± 2.4	15.0 ± 1.3 (28)
	HRBC	20.3 ± 4.7	21.9 ± 6.0	20.0 ± 3.8 (0)

* Time courses of transmembrane equilibration of 500 μM [^{14}C]Hyp (0.5 cpm/pmole) and 500 μM [^{14}C]Ade (0.4 cpm/pmole) were measured by rapid kinetic techniques in samples of suspensions of 5.5×10^6 N1S1-67 cells, 8.6×10^6 CHO cells, 1.8×10^7 L1210 cells, 2.4×10^7 P388 cells and 4.5×10^8 human red blood cells (HRBC)/ml, which were supplemented with the indicated concentrations of dilazep. Initial entry velocities were computed by integrated rate analysis assuming directional symmetry and equal mobility of loaded and empty carrier [19, 27].

† Percent inhibition by 100 μM dilazep.

hypoxanthine transport in L1210 and P388 cells and in human erythrocytes, even though uridine transport in these cells was much more sensitive to inhibition by dilazep than uridine transport in NISI-67 and CHO cells (Talble 1). Hypoxanthine transport in the cultured cell lines exhibits the same differential sensitivity to inhibition by dipyridamole [27]. Adenine transport was relatively little affected by dilazep in any of the cell lines, although the inhibition in Novikoff cells seems significant (Table 2). This finding lends support to the view that adenine is transported by a carrier different from those transporting nucleosides and hypoxanthine [1].

We have also measured the uptake of [^{14}C]hexobendine by various types of cells. In human erythrocytes, 22.5 μM [^{14}C]hexobendine equilibrated with the intracellular space within 2 sec of incubation at 25° (Fig. 7A). In contrast, the amounts of [^{14}C]hexobendine accumulated by CHO and P388 cells (Fig. 7A), as well as L1210 cells (Fig. 7B) after 2 sec of incubation, exceeded that in the medium 3- to 4-fold, and there was additional uptake at a slow rate for 2–4 min of incubation to levels up to 10-fold the medium concentration. Similarly, within 3 min of intravenous injection of [^{14}C]hexobendine into rats, it was recovered at much higher levels in various

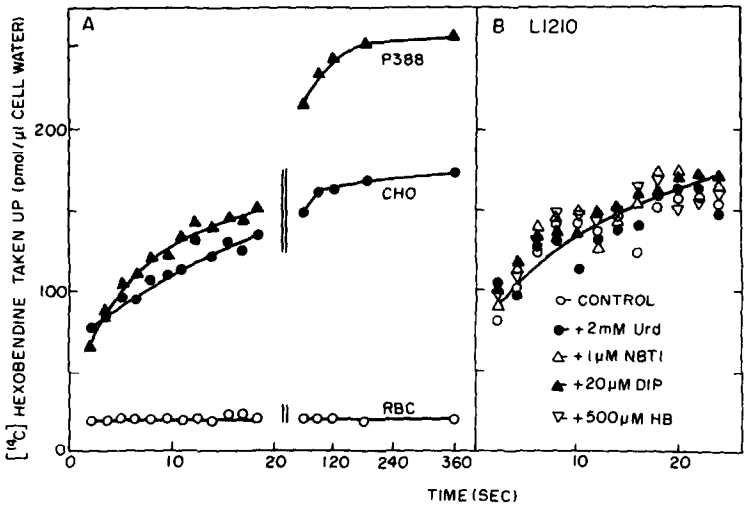


Fig. 7. Uptake of [^{14}C]hexobendine by human red blood cells and various cultured cells at 25°. The uptake of 22.5 μM [^{14}C]hexobendine (16 cpm/pmole) was measured by rapid kinetic techniques in suspensions of (A) 6×10^8 human erythrocytes/ml, 1.6×10^7 P388 cells/ml, and 7×10^6 CHO cells/ml or (B) 2.4×10^7 L1210 cells/ml. Where indicated in (B), samples of the suspension of L1210 were supplemented with uridine (Urd), dipyridamole (DIP), NBTI or unlabeled hexobendine (HB) to final concentrations of 2 mM, 20 μM, 1 μM and 500 μM, respectively, 1–2 min before the uptake of [^{14}C]hexobendine was measured.

tissues than in plasma [24]. The concentrative accumulation of [^{14}C]hexobendine in the cell lines cannot be accounted for by its solubilization in cellular lipids. We determined the octanol/H_2O partition coefficient (K_{oct}) of hexobendine to be about 8.0. We have shown previously that K_{oct} yields a good estimate of the solubility of substances in the lipids of cultured cells [25]. On the basis of a lipid content of about 10 μg/μl cell water*, we estimate that only 2 pmoles of [^{14}C]hexobendine would be maximally dissolved in membrane lipids per μl cell water in the experiments illustrated in Fig. 7. Concentrative accumulation of radioactivity from [^{14}C]hexobendine also did not result from metabolic conversions of hexobendine (see below). Thus, we conclude that cultured cells, but not human red blood cells, possess components that bind hexobendine. Whether binding occurs at the cell surface or is to intracellular components cannot be decided on the basis of the present data, although its lipophilicity indicates that hexobendine should equilibrate across the plasma membrane in less than 1 min. This binding must be unrelated to the inhibition of nucleoside transport, since uridine transport of human erythrocytes was at least as sensitive to inhibition by hexobendine as that of any of the cultured cell line and the extent of [^{14}C]hexobendine binding to the cell lines was unrelated to the sensitivity of uridine transport in these cells to hexobendine inhibition (cf. Figs. 3 and 7). Furthermore, the potency of inhibition of uridine transport by hexobendine was about the same whether the inhibitor was added to cells simultaneously with uridine or 2–10 min prior to the transport assay (data not shown). This finding indicates that the inhibitor interacts very rapidly with the transporter. In addition, the data in Fig. 7B show that the concentrative accumulation of [^{14}C]hexobendine by L1210 cells was not affected significantly by the presence of 500 μM unlabeled hexobendine or by uridine, NBTI or dipyridamole. Uptake of [^{14}C]hexobendine by human erythrocytes was also not affected by these substances (data not shown). This finding lends further support to the view that the inhibition of nucleoside transport by the various inhibitors that are structurally unrelated to nucleosides is not simply due to a competition between inhibitor and substrate for the substrate binding site of the transporter, even though the inhibition of transport is of an apparent competitive type [2, 3] or of a mixed type with the major effect being on the apparent K_m of nucleoside transport [1]. Furthermore, the data indicate that in the concentration range analyzed (20–500 μM) [^{14}C]hexobendine binding to cells was non-saturable. The relatively low specific radioactivity of the [^{14}C]hexobendine precluded investigating the potential presence of saturable high-affinity binding sites (with K_d values in the nanomolar range).

Dilazep and hexobendine have been shown to be metabolized in rats [24, 28]. Metabolism after intravenous injection involves cleavage of the ester bonds of hexobendine and demethylation, but it is relatively slow and seems to be confined to the liver and perhaps the kidney. We have not detected a significant degradation of 30–70 μM [^{14}C]hexobendine in suspensions of 1×10^7 P388 or 5×10^8 human erythrocytes/ml during 3 hr of incubation at 25°. Samples of cells were collected at various times of incubation with [^{14}C]hexobendine and extracted with 0.5 N perchloric acid, 1 mM EDTA at 100°, or methanol, and the cell extracts and culture fluid samples were chromatographed on two solvent systems (see Materials and Methods). About 90% of the radioactivity of [^{14}C]hexobendine comigrated with hexobendine (R_f about 0.95 in the *n*-butanol/2-butanone/acetone/NH_4OH/H_2O solvent, see Ref. 24) whether or not it had been incubated with cells; 5–10% of radioactivity was recovered in a fraction with an R_f of about 0.5 in the above solvent system, but there was no significant change in this fraction during incubation with cells. Also, the absorption spectrum of hexobendine in the culture fluid remained essentially unaltered during incubation with cells (data not shown). Furthermore, we have incubated 40 nM hexobendine in balanced salt solution and a suspension of 1.7×10^7 P388 cells/ml of balanced salt solution containing 40 nM hexobendine at 25°. At various times of incubation, the cells of samples of suspension were removed by centrifugation at 8000 *g* for 10 min, and the supernatant fraction as well as the solution of 40 nM hexobendine were assayed for inhibition of equilibrium exchange of 500 μM uridine (see Fig. 3). Uridine equilibrium exchange was inhibited 87–89% and 78–81% by the 40 nM hexobendine and the culture fluid from the P388 cell suspension incubated with 40 nM hexobendine respectively. The inhibition by the culture fluid from P388 cells was about the same whether it was obtained within 2 min after addition of hexobendine to the cell suspension or after 3 hr of incubation. The slightly lower inhibition by the culture fluid from the P388 cells than by the untreated 40 nM hexobendine can readily be accounted for by the concentrative accumulation of hexobendine by the cells and the consequent lowering of the hexobendine concentration in the culture fluid.

Figure 8 compares the effects of dipyridamole, dilazep and hexobendine on the growth of various cell lines. Dipyridamole inhibited the growth of all cell lines at concentrations >10 μM, but CHO cells seemed to be the least affected. A similar growth inhibition by dipyridamole has been reported recently for two human tumor cell lines [29] and a mouse hepatoma line [30]. Growth of the various cell lines was slightly more resistant to dilazep than to dipyridamole, and even more so to hexobendine. These results, coupled with the finding that dilazep and hexobendine are more effective in inhibiting uridine transport than dipyridamole, at least in some cell lines, suggest that they might be effective in limiting the growth of nucleoside-dependent cells at non-cytotoxic concentrations. A preliminary experiment with S49 cells supports this conclusion. The data in Table 3 show that the growth inhibition of S49 cells by treatment with methotrexate or pyrazofurin was reversed completely by the presence of thymidine plus hypoxanthine, or of uridine, respectively (first column). The additional presence of 1 μM

* This calculation is based on a lipid content of Novikoff rat hepatoma cells of 250 μg/10^7 cells [25] and an intracellular water volume of 25 μl/10^7 cells [5].

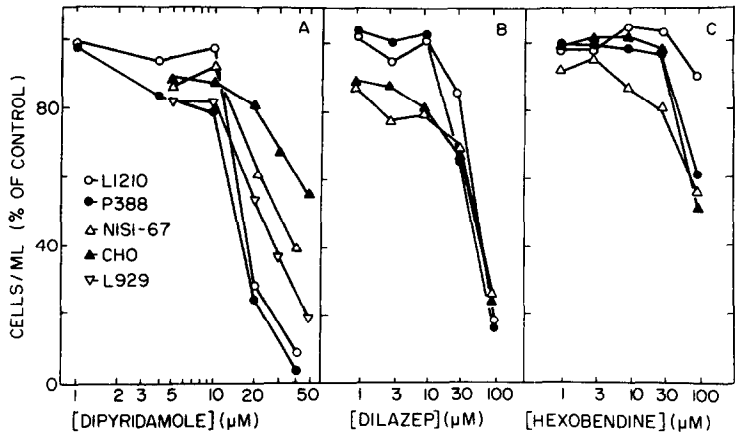


Fig. 8. Effects of dipyridamole (A), dilazep (B) and hexobendine (C) on the growth of various cell lines. Twenty-four-well tissue culture plates containing the indicated concentrations of dipyridamole, dilazep or hexobendine were seeded with 1–2 × 10^5 cells/well and incubated at 37° in a CO_2 incubator for 3 days and then the cells were enumerated. Control cultures of the various cell types contained between 0.7 and 1.5 × 10^6 cells/well.

NBTI was effective in preventing this reversal in S49 cells (second column), because these cells express only NBTI-sensitive transport (see Table 1). The presence of 10 μM dipyridamole or 40 μM hexobendine was equally effective in inhibiting the growth of the nucleoside-dependent cells (columns 3 and 4). Dipyridamole exhibited considerable toxicity at this concentration, but 40 μM hexobendine by itself had relatively little effect on cell growth.

Some inhibitors of *de novo* nucleotide synthesis are useful anticancer agents but their effectiveness might be limited by the salvage of nucleosides and nucleobases from the blood by the tumor cells. It has, therefore, been suggested that co-administration of nucleoside transport inhibitors may enhance the potency of such anticancer agents *in vivo*. Our data suggest that hexobendine may be more useful as an inhibitor of the growth of nucleoside-dependent tumor cells than dipyridamole, which has been found to only partially inhibit the growth of such cells at non-toxic concentrations [11, 30]. NBTI is also of limited use in this respect [31], because the NBTI-resistant nucleoside transport activity exhibited by most cells [5, 7] is probably adequate for their salvage needs.

Table 3. Effects of transport inhibitors on the growth of S49 cells made dependent on nucleosides by treatment with methotrexate or pyrazofurin*

Treatments	Cells/well (% of control) + transport inhibitor			
	None	NBTI	DIP	HXB
MTX	33	ND†	ND	ND
MTX + dThd/Hyp	105	41	30	33
PYF	24	ND	ND	ND
PYF + Urd	100	38	37	37
None	100	103	53	82

* Wells of 24-well tissue culture plates received 1 ml of a suspension of 1.5 × 10^5 S49 cells/ml, and where indicated, 1 μM methotrexate (MTX), 1 μM pyrazofurin (PYF), 20 μM thymidine (dThd) plus 200 μM hypoxanthine (Hyp), 100 μM uridine, 1 μM NBTI, 10 μM dipyridamole (DIP), or 40 μM hexobendine (HXB). The plates were incubated at 37° for 3 days and the cells enumerated. Cell counts are averages of duplicate wells and are expressed as percent of control wells, which contained 1.22 × 10^6 cells.

† ND = not determined.

The degree of growth inhibition of nucleoside-independent cells by the various substances (Fig. 8) was not related to their potency as inhibitors of nucleoside transport. This finding, coupled with the fact that hexobendine inhibited the growth of nucleoside-dependent but not of control S49 cells (Table 3), makes it unlikely that growth inhibition of the nucleoside-independent cells is mediated by an inhibition of nucleoside transport. However, it could be a consequence of an inhibition of the transport of another substrate essential for growth. Dipyridamole, for example, seems to have a broad effect on facilitated diffusion systems, inhibiting also sugar and phosphate transport (see Ref. 1). On the other hand, growth inhibitions could be due to a general membrane perturbation resulting from an interaction of these lipophilic substances with components of the cell membrane or to an interaction with other cellular components. A general perturbation of the plasma membrane is suggested by the finding that high concentrations of dipyridamole also inhibit the non-mediated permeation of hydrophilic substances, such as L-glucose and cytosine [25]. The finding that hexobendine effectively inhibited the growth of nucleoside-dependent S49 cells (Table 3) also supports the conclusion that degradation of hexobendine by cells was not responsible for its relatively low general cytotoxicity when compared to that of dipyridamole.

The molecular basis of the inhibition of facilitated transport by various lipophilic substances is unclear, but most likely also reflects a general interaction with membrane components including integral membrane proteins, such as the transport carriers. The $\log_{10}$

IC_{50} values for the inhibition of uridine transport in Novikoff cells by a number of inhibitors were found to be inversely proportional to their lipid solubility [32], but the present study shows that hexobendine falls outside this relationship. Such a relationship could also not explain the marked differential sensitivities of nucleoside and hypoxanthine transport to inhibition by dipyridamole and dilazep in various cell lines (Tables 1 and 2), and why the patterns of sensitivity differ for nucleoside and hypoxanthine transport. Nevertheless, it is clear that, whenever hypoxanthine transport in a cell line is inhibited by dipyridamole, and presumably by dilazep, it is also strongly inhibited by nucleosides, and nucleoside transport is inhibited by hypoxanthine, whereas this reciprocal inhibition of nucleoside and hypoxanthine transport by each other is not observed in cells, such as L1210 and P388 cells and human erythrocytes, whose hypoxanthine transport is highly resistant to dipyridamole or dilazep (see Table 2 and [27]). Knowledge of the structure of the transporters and their conformation in the plasma membrane should greatly facilitate an understanding of their molecular function and interaction with various inhibitors.

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