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Membrane Permeation Characteristics of 5′-Modified Thymidine Analogs

BARBARA A. DOMIN, WILLIAM B. MAHONY, GEORGE W. KOSZALKA, DAVID J. T. PORTER, GERALD HAJIAN, and THOMAS P. ZIMMERMAN

Divisions of Experimental Therapy and Toxicology (G.H.), Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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SUMMARY

The membrane permeation characteristics of 5′-deoxythymidine (5′-ddThd) and 5′-azido-5′-deoxythymidine (5′-N_3-5′-ddThd) were investigated in human erythrocytes, with an inhibitor-stop assay, at 20°. Uptake of both nucleoside analogs occurred without metabolism, was nonconcentrative, and was partially inhibited by nucleosides or inhibitors of nucleoside transport at micromolar permeant concentrations. At higher permeant concentrations (>1.0 mM), the influx rate of each analog was linearly dependent on concentration and insensitive to inhibition by nucleosides, inhibitors of nucleoside transport, and nucleobases. Kinetic analyses using nonlinear regression revealed that a saturable component of 5′-ddThd influx (K_m = 200 μM) was competitively inhibited by thymidine (dThd) (K_i = 86 μM) or 5-iodo-2′-deoxyuridine (K_i = 84 μM). Similarly, a saturable component of 5′-N_3-5′-ddThd influx (K_m = 220 μM) was competitively inhibited by 2-chloroadenosine (K_i = 18 μM). The K_i values for these nucleoside inhibitors were similar to their reported K_m values as permeants of the nucleoside transporter. Both 5′-ddThd and 5′-N_3-5′-ddThd competitively inhibited the influx of dThd (K_m = 60 μM), with similar K_i values (150 and 200 μM, respectively). We conclude that these two 5′-modified dThd analogs enter human erythrocytes both by nonfacilitated diffusion and by the nucleoside transporter. The absence of the 5′-hydroxyl group of dThd (5′-ddThd) resulted in a large increase in the octanol/buffer partition coefficient, in an ability to permeate human erythrocytes by nonfacilitated diffusion, and in a 3-fold diminished binding to the nucleoside transporter. The 5′-azido group (5′-N_3-5′-ddThd) resulted in an additional 1.4-fold increase in the octanol/buffer partition coefficient and in a 2-fold increase in the rate of nonfacilitated diffusion.

Antiviral 2′,3′-dideoxynucleosides enter cells by the unusual mechanism of nonfacilitated diffusion. Compounds that depend mainly on nonfacilitated diffusion for cellular entry are 3′-N_3-3′-ddThd (1), 3′-ddThd (2), 2′,3′-dideoxyadenosine (3–5), 2′,3′-dideoxyinosine (3), 2′,3′-dideoxyguanosine (6), 2′-fluoro-2′,3′-dideoxyarabinosyladenine (5), 3′,5′-trideoxythymidine (4), and 3′-deoxythymidin-2′-ene (7). A partial dependence on nonfacilitated diffusion for permeation has been described for 2′,3′-dideoxyadenosine (8) and 2′,3′-dideoxycytidine (9–13). The observation that nonfacilitated diffusion is a major pathway for permeation of cell membranes by the dideoxynucleoside analogs has been related both to the lack of affinity for the nucleoside transporter, resulting from the absence of the 3′-OH group, and to an increased lipophilicity (1, 2, 7, 11). The contrast in transport mechanisms between these antiviral dideoxynucleosides (largely nonfacilitated diffusion) and physiological nucleosides (carrier-mediated influx) provides a rationale for potentiation of the antiviral activity of dideoxynucleosides by inhibition of the salvage pathway with inhibitors of nucleoside transport (1, 14–16). In order to better understand the determinants for permeation via nonfacilitated diffusion versus those for transport via the nucleoside carrier, we have investigated the mechanisms of entry of 5′-ddThd and 5′-N_3-5′-ddThd (Fig. 1) into human erythrocytes. The results of this study show that these 5′ modifications of dThd affect transport differently than do their 3′ counterparts.

Experimental Procedures

Materials. 5′-ddThd and 5′-N_3-5′-ddThd were synthesized by Dr. Susan Daluge of these laboratories.

Synthesis of [*methyl*-^{3}H]5′-ddThd. [*methyl*-^{3}H]Thymine (1 mCi, 65 Ci/mol; Amersham International) and 5′-ddThd (0.17 mg) were dissolved in 0.125 ml of potassium phosphate buffer (10 mM, pH 7.4). The reaction was initiated with 40 units (0.01 ml) of purified *Escherichia coli* dThd phosphorylase (17), and the solution was incubated at 25° for 120 min. Incorporation of the label into the nucleoside was followed by chromatography on silica gel TLC plates developed in chloroform/methanol (9:1). The R_F of 5′-ddThd was 0.31 in this mobile phase. [^{3}H]5′-ddThd was purified by C_{18} reverse phase HPLC, with a mobile phase of acetonitrile/water (9:1). The fractions containing [^{3}H]

ABBREVIATIONS: 3′-N_3-3′-ddThd, 3′-azido-3′-deoxythymidine; 5′-ddThd, 5′-deoxythymidine; dThd, thymidine; 3′-ddThd,3′-deoxythymidine; 5′-N_3-5′-ddThd, 5′-azido-5′-deoxythymidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IdUrd, 5-iodo-2′-deoxyuridine; NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

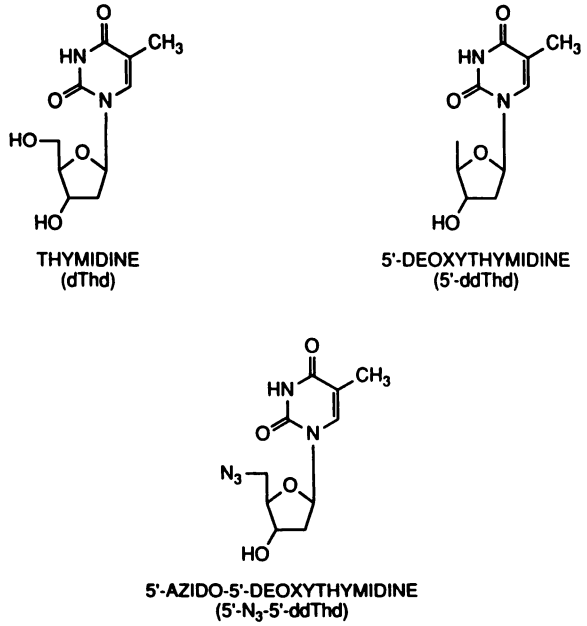

Fig. 1. Structures of dThd analogs.

5′-ddThd were combined and, after evaporation of the solvent, the product was dissolved in 95% ethanol (2 ml). The final radiochemical purity was >99%, and the specific activity of this [^{3}H]5′-ddThd was 0.54 Ci/mmol.

Synthesis of [*methyl*-^{3}H]5′-N_3-5′-ddThd. [*methyl*-^{3}H]Thymine (5 mCi, 48 Ci/mmol; Amersham International) was combined with 5′-N_3-5′-ddThd (3 mg) and 100 units of *E. coli* dThd phosphorylase (17), in 10 ml of potassium phosphate buffer (pH 7.0), at 37°. 5′-N_3-5′-ddThd (R_F = 0.34) was separated from thymine (R_F = 0.26) on silica gel TLC plates developed with chloroform/methanol (8:2), and incorporation of the radiolabel was monitored with the aid of a Bioscan TLC scanner. On days 2 and 7 an additional 400 units of enzyme were added, and on day 11 the solvent was evaporated from the reaction. [^{3}H]5′-N_3-5′-ddThd was purified by chromatography through three consecutive C_{18} reverse phase columns (10 × 250 mm; 5-μm particle size), with decreasing concentrations of acetonitrite in water, i.e., 25%, 20%, and 18% acetonitrile. The final radiochemical purity was >98%, and the specific activity was 0.50 Ci/mmol.

[U-^{14}C]Sucrose (4 mCi/mmol) and [^{3}H]water (1 mCi/g) were from DuPont-New England Nuclear. 2-Chloroadenosine was from Aldrich Biochemicals, and other unlabeled nucleobases and nucleosides, papaverine hydrochloride, dipyridamole, and NBMPR were from Sigma. Dilazep was kindly provided by Hoffmann-LaRoche, and HEPES was from GIBCO.

Preparation of human erythrocytes. Human erythrocytes were collected from healthy volunteers and were prepared as described previously (1, 18).

Kinetics of 5′-ddThd and 5′-N_3-5′-ddThd influx. Assays of 5′-ddThd and 5′-N_3-5′-ddThd influx were performed at 20° in 10 mM HEPES-saline buffer (pH 7.3), using the "papaverine-stop" method, as described previously (18). Initial velocities of influx were calculated by linear regression analysis of the slopes of plots of cell-associated radioisotope versus assay time during the linear phase of influx.

"Oil-stop" assays (19) of 5′-ddThd and 5′-N_3-5′-ddThd influx were performed with the modifications described previously (2, 18). The assay termination was defined as the starting time of the microcentrifugation. The amount of extracellular radioactivity in the cell pellet was determined using [^{14}C]sucrose (1).

Kinetics of dThd influx. Assays of dThd influx were at 20°, as described previously for IdUrd influx (20).

Metabolism studies. Cells were incubated for 20 min with 1 μM or 12 mM [^{3}H]5′-ddThd or [^{3}H]5′-N_3-5′-ddThd, under the same conditions used for influx measurements. Each incubation was terminated with 700 μl of ice-cold papaverine, and the cell-associated radioactivity was extracted with cold trichloroacetic acid, as described previously (18), and analyzed by reverse phase HPLC (21).

Data analysis. Kinetic constants were determined by fitting the data by nonlinear regression (22), with one of the following equations, using a $1/V^2$ weighting factor (23):

$$v = \frac{(V_{max})\ (S)}{S + K_m} + (c)\ (S) \quad (1)$$

for competitive inhibition

$$v = \frac{(V_{max})\ (S)}{S + K_m(1 + I/K_i)} + (c)\ (S) \quad (2)$$

or, for noncompetitive inhibition

$$v = \frac{(V_{max})\ (S)}{S(1 + I/K_i) + K_m(1 + I/K_i)} + (c)\ (S) \quad (3)$$

The c term is the rate constant for nonfacilitated diffusion. p values for competitive versus noncompetitive inhibition were obtained by use of an approximate F test, using residual sum of squares from the nonlinear regression analyses (24). Statistical comparisons of influx rates were determined with the PROC GLM computer program from SAS Institute, Inc. (25).

Results

Time dependence of influx. The oil-stop assay was used to determine the time dependence of the influx of 500 μM [^{3}H]5′-ddThd (Fig. 2A) and of 550 μM [^{3}H]5′-N_3-5′-ddThd (Fig. 2B) at 20°. Equilibration of each permeant inside the cells was attained within 9 min. At equilibrium, the intracellular concentrations (2100 pmol/ξ μl of packed cells for 5′-ddThd, 2700 pmol/5 μl of packed cells for 5′-N_3-5′-ddThd) were approximately equal to the respective extracellular concentrations.

The amount of cell-associated permeant at the earliest assay time was approximately 10% of the equilibrium value with these high permeant concentrations. Influx generally proceeds linearly with time until the intracellular permeant concentration approaches values of ~20% of equilibrium (26). Because the time of linear uptake would be shorter at subsaturating permeant concentrations, influx measurements with the oil-stop method might be inadequate. We therefore investigated the use of the papaverine-stop assay (18) for these compounds.

Cold papaverine as a "stopper" for the influx of 5′-ddThd and 5′-N_3-5′-ddThd. The influx of 12 mM [^{3}H]5′-ddThd (Fig. 3A) and 9.1 mM [^{3}H]5′-N_3-5′-ddThd (Fig. 3B) was measured using the papaverine-stop assay, which was shown previously to be effective in measuring both nucleobase (18) and nucleoside (20) influx. At these concentrations, the influx of both permeants was linear with time for >15 sec, and influx rates obtained with the papaverine-stop were similar to those obtained with the oil-stop.

Criteria for complete and instantaneous stopping of influx by the papaverine-stop consisted of 1) a similar value for cell-associated permeant at time 0, in comparison with the [^{14}C]sucrose space (Fig. 3), and 2) little or no change in influx after the addition of the cold papaverine solution. The cell-associated radioactivity at time 0 for [^{3}H]5′-ddThd (0.05% of total) was similar to the [^{14}C]sucrose space (0.04% of total), whereas the time 0 cell-associated [^{3}H]5′-N_3-5′-ddThd value (0.07% of total) was somewhat higher than the sucrose space. Varying the time between the addition of the cold papaverine solution and

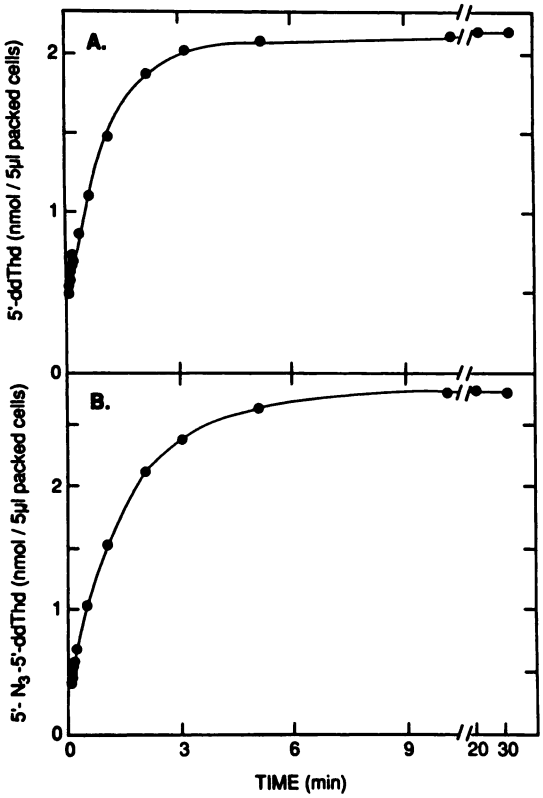

Fig. 2. Time dependence of influx. Human erythrocytes (5 µl of packed cells) were incubated at 20° in HEPES-saline buffer (pH 7.3), for the indicated times, with 500 µM [^{3}H]5′-ddThd (3.3 Ci/mol) (A) or 550 µM [^{3}H]5′-N$_3$-5′-ddThd (4.0 Ci/mol) (B), in a total volume of 100 µl. Assays were terminated with the oil-stop method, and each time point is the mean ± standard error of triplicate values. Error bars were omitted where they did not extend beyond the symbol boundaries.

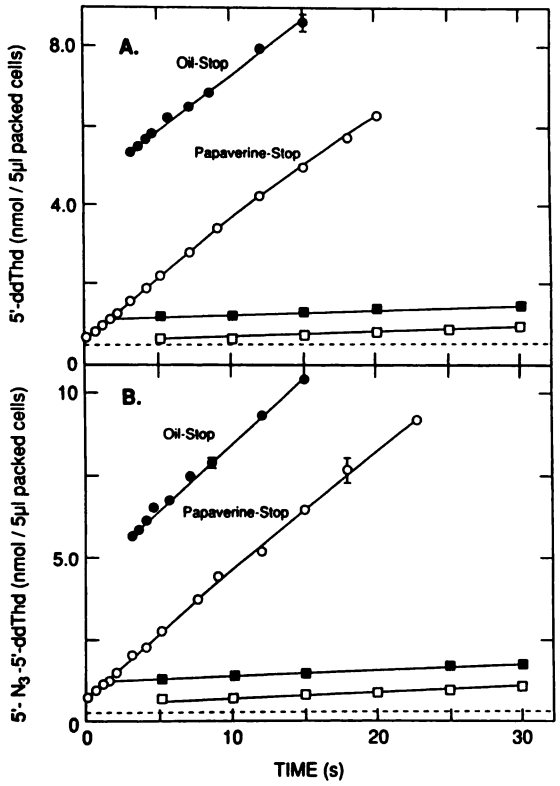

Fig. 3. Efficacy of a cold papaverine solution as a stopper for influx of 5′-ddThd or 5′-N$_3$-5′-ddThd. Human erythrocytes (5 µl of packed cells) were incubated at 20° in HEPES-saline buffer (pH 7.3) with 12 mM [^{3}H] 5′-ddThd (0.14 Ci/mol) (A) or 9.1 mM [^{3}H]5′-N$_3$-5′-ddThd (0.24 Ci/mol) (B), in a total volume of 100 µl. The oil-stop method (●) used the initiation of centrifugation as the assay termination time, with no attempt to correct for the time required for sedimentation of the cells into the oil phase. The papaverine-stop method (○) used the addition of 700 µl of cold 19 mM papaverine solution as the assay termination time, and centrifugation of the cells through oil was initiated 10 sec after the papaverine addition. For both of these conditions, the time indicated in the graph is the assay termination time. The two conditions used for determining the efficacy of the assay termination were as follows. 1) Cells were incubated for 1.5 sec with the ^{3}H-labeled permeant. The cold papaverine solution was then added, and cells were kept at room temperature, before centrifugation, for 10 sec **plus** the additional times indicated in the graph (■). 2) The cold papaverine solution was added to the cells before the ^{3}H-labeled permeant, and the cells were kept at room temperature, before centrifugation, for the times indicated in the graph (□). – – –, [^{14}C]Sucrose space for each experiment. Each time point is the mean ± standard error of triplicate values, and error bars were omitted when they did not extend beyond the symbol boundaries.

the centrifugation of the cells for both a 1.5-sec (Fig. 3) and a time 0 (Fig. 3) assay revealed small residual rates of influx for both permeants in the presence of the cold papaverine solution.

The effect of this small leakage rate on the calculations of permeant influx rates was assessed with 5′-N$_3$-5′-ddThd, the permeant with the greater leakage rate. The effects of varying the times before centrifugation of the cells (delay times) on initial velocities of influx of 10 mM [^{3}H]5′-N$_3$-5′-ddThd were examined (Table 1). The measured initial rate of influx was unchanged with delay times from 5 to 20 sec. However, the time 0 values at ≥20-sec delay times were significantly different from that at a 5-sec delay time. Thus, identical rate curves could be obtained only when the cells were centrifuged within 15 sec of the cold papaverine solution addition, and this condition was maintained throughout this study.

Metabolism. Metabolism of 1.0 µM or 12 mM [^{3}H]5′-ddThd and 1.8 µM or 12 mM [^{3}H]5′-N$_3$-5′-ddThd was examined after 20-min incubations with human erythrocytes at 20°. In each case, >95% of the cell-associated radioactivity was eluted from the reverse phase HPLC column, with the same retention times as those of the authentic standards for these two compounds.

Partition coefficients. The partition coefficients for 5′-ddThd and 5′-N$_3$-5′-ddThd were determined, by the classical shake-flask technique (in 1-octanol/phosphate buffer, pH 7.0), to be 0.71 and 1.0, respectively.

Effects of various compounds on the rate of influx of 5′-ddThd and 5′-N$_3$-5′-ddThd. Nucleobases, nucleosides, dideoxynucleosides, and inhibitors of nucleoside transport were examined as inhibitors of the influx of [^{3}H]5′-ddThd (0.9 µM and 5.0 mM) or [^{3}H]5′-N$_3$-5′-ddThd (1.0 µM and 5.0 mM) (Table 2). The influx of both permeants at low concentrations was substantially inhibited (52–94% inhibition) by nucleosides (dThd and uridine) and by inhibitors of nucleoside transport (dilazep, NBMPR, and dipyridamole). 3′-ddThd, 3′-N$_3$-3′-ddThd, 5′-ddThd, and 5′-N$_3$-5′-ddThd were also inhibitory (19–80% inhibition) at the low permeant concentrations. Adenine and hypoxanthine inhibited by ≤24%. At high permeant concentrations (5.0 mM), the influx of both permeants was inhibited slightly (<20%), or not at all, by nucleosides or inhibitors of nucleoside transport.

Concentration dependence of influx rate. Because the influx of both permeants was inhibited by nucleosides or inhib-

TABLE 1

Effect on 5′-N_3-5′-ddThd influx rate of the time between addition of cold 19 mM papaverine and centrifugation of erythrocytes through oil

The initial velocity of influx of 10 mM [^{3}H]5′-N_3-5′-ddThd (0.18 Ci/mol) into human erythrocytes was determined at 20°, as described in Experimental Procedures. Initial velocities (mean ± standard error) were derived by linear regression analysis of data from triplicate assays conducted for 0, 2.0, 4.0, 6.0, and 8.0 sec and with intervals of 5–30 sec between the time of addition of cold 19 mM papaverine to the influx assay mixture and centrifugation of the cells through oil.

Time between addition of cold papaverine and centrifugation	Influx rate of 5′-N_3-5′-ddThd
sec	*nmol/sec/5 μl of packed cells*
5.0	0.43 ± 0.02
10	0.41 ± 0.01
15	0.44 ± 0.01
20	0.42 ± 0.01
30	0.39 ± 0.01[a]

[a] Statistically different from the rate obtained with a 5-sec interval between papaverine addition and centrifugation, $p < 0.05$.

TABLE 2

Effects of nucleobases, nucleosides, and inhibitors of nucleoside transport on the rate of influx of 5′-ddThd and 5′-N_3-5′-ddThd into human erythrocytes

The initial velocity of influx of each labeled permeant was determined at 20°, as described in Experimental Procedures. Compounds were added simultaneously with the radiolabeled permeants except where indicated. Initial velocities were derived by linear regression analysis of data obtained during the linear phase of influx. Values obtained when the same analog (unlabeled) was the additive are uncorrected for changes in specific activities. No correction was made for the contribution of nonfacilitated diffusion, which was calculated to be 8% (0.9 μM) or 86% (5.0 mM) for 5′-ddThd and 44% (1.0 μM) or 100% (5.0 mM) for 5′-N_3-5′-ddThd.

Additive	Inhibition of influx[a]			
	[^{3}H]5′-ddThd		[^{3}H]5′-N_3-5′-ddThd	
	0.9 μM[b]	5.0 mM[c]	1.0 μM[d]	5.0 mM[e]
	%			
Adenine (1.0 mM)	24[f]	0	18[g]	0
Hypoxanthine (1.0 mM)	17[g]	3	16[g]	0
Guanine (94 μM)	1	6	0	0
Thymidine (1.0 mM)	85[f]	11[g]	55[f]	0
Uridine (1.0 mM)	86[f]	8[h]	52[f]	0
Dilazep (1.0 μM)[i]	93[f]	20[f]	61[f]	4
NBMPR (1.0 μM)[i]	93[f]	16[f]	61[f]	3
Dipyridamole (1.0 μM)[i]	94[f]	19[f]	62[f]	6
3′-ddThd (1.0 mM)	29[f]	4	19[f]	0
5′-ddThd (1.0 mM)	80[f]	4	50[f]	0
3′-N_3-3′-ddThd (1.0 mM)	59[f]	0	37[f]	0
5′-N_3-5′-ddThd (1.0 mM)	78[f]	0	49[f]	0

[a] Assay times were as follows: 0, 0.5, 1.0, and 1.5 sec for 0.9 μM [^{3}H]5′-ddThd (540 Ci/mol); 0, 2.0, 4.0, and 6.0 sec for 5.0 mM [^{3}H]5′-ddThd (0.56 Ci/mol); 0, 2.0, 4.0, 5.0, 6.0 sec for 1.0 μM [^{3}H]5-N_3-5′-ddThd (500 Ci/mol); and 0, 2.0, 4.0, 6.0, and 7.0 sec for 5.0 mM [^{3}H]5′-N_3-5′-ddThd (0.45 Ci/mol).
[b] Control rate was 0.31 ± 0.01 pmol/sec/5 μl of packed cells.
[c] Control rate was 140 ± 2 pmol/sec/5 μl of packed cells.
[d] Control rate was 0.10 ± 0.002 pmol/sec/5 μl of packed cells.
[e] Control rate was 194 ± 3 pmol/sec/5 μl of packed cells.
[f] Significantly different from control value, $p < 0.001$.
[g] Significantly different from control value, $p < 0.01$.
[h] Significantly different from control value, $p < 0.05$.
[i] Cells were preincubated with inhibitor for 20 min before addition of radiolabeled permeant.

itors of nucleoside transport, the concentration dependence of their influx rates was examined in the absence or presence of 1.0 μM dilazep (Fig. 4A, 5′-ddThd; Fig. 4B, 5′-N_3-5′-ddThd). In the absence of dilazep, the influx rate of 5′-ddThd increased with increasing concentration in a markedly biphasic manner. There was evidence of rate saturability at concentrations of <1.0 mM and a linear dependence of influx rate on concentration at higher concentrations (indicative of nonfacilitated diffusion). The influx rate was linearly dependent on concentration in the presence of dilazep.

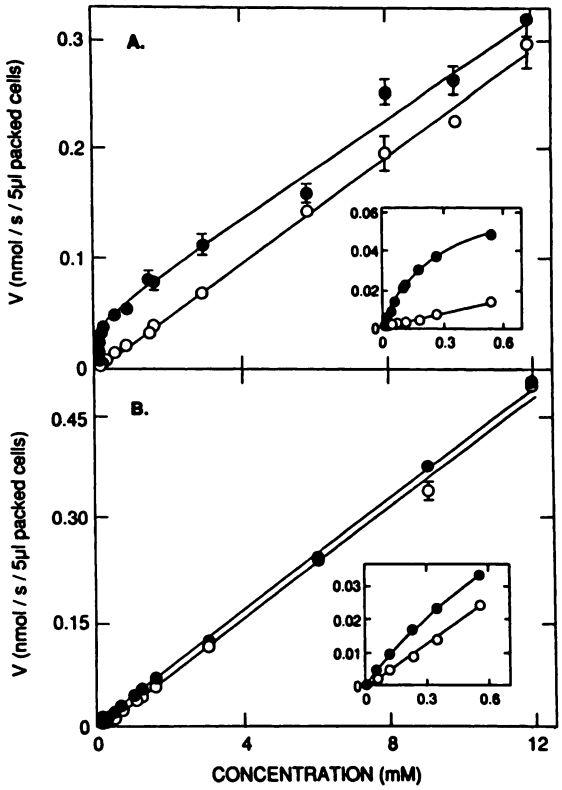

Fig. 4. Concentration dependence of 5′-ddThd and 5′-N_3-5′-ddThd influx. Assays were performed at 20° in the absence (●) or presence (○) of 1.0 μM dilazep (preincubated with the cells for 20 min before permeant addition), as described in Experimental Procedures. Permeant concentrations were 13 μM to 12 mM [^{3}H]5′-ddThd (0.30 or 40 Ci/mol) (A) and 5 μM to 12 mM [^{3}H]5′-N_3-5′-ddThd (0.45 or 66 Ci/mol) (B). Velocities were determined by linear regression analysis of data obtained during the linear phase of influx (0, 0.5, 1.5, and 2.0 sec for 5′-ddThd influx and 0, 1.0, 2.0, 4.0, 6.0, and 8.0 sec for 5′-N_3-5′-ddThd influx). Error bars represent the standard errors of the slopes obtained with this analysis, and these were omitted where they did not extend beyond the symbol boundaries. The curve in the absence of dilazep is that derived by nonlinear regression analysis of the data using eq. 1, given in Experimental Procedures. The line in the presence of dilazep is that derived by linear regression analysis of the data. *Inset*, data at permeant concentrations of <0.6 mM.

Qualitatively, the same profile was evident for 5′-N_3-5′-ddThd influx. In the absence of dilazep, the saturable portion of the curve could be detected only at low concentrations (Fig. 4B, *inset*), whereas the linear dependence of influx rate on concentration was predominant over the entire concentration range. At concentrations of >2.0 mM, the influx was not measurably inhibited by dilazep.

Data from both experiments (in the absence of dilazep) were fitted to eq. 1 by nonlinear regression, to give estimates for K_m, V_{max}, and a nonfacilitated diffusion rate constant as follows: 160 ± 20 μM, 49 ± 3 pmol/sec/5 μl of cells, and 22 ± 1 pmol/sec/mM/5 μl of cells, respectively, for 5′-ddThd, and 200 ± 20 μM, 14 ± 1 pmol/sec/5 μl of cells, and 39 ± 1 pmol/sec/mM/5 μl of cells, respectively, for 5′-N_3-5′-ddThd.

Inhibition of 5′-ddThd and 5′-N_3-5′-ddThd influx by permeants of the nucleoside transporter. Data from inhi-

bition studies were fitted by nonlinear regression to eqs. 2 and 3 (Fig. 5A, dThd inhibition of 5′-ddThd influx; Fig. 5B, 2-chloroadenosine inhibition of 5′-N_3-5′-ddThd influx). p values for competitive inhibition versus noncompetitive inhibition were 0.999 for dThd and 1.00 for 2-chloroadenosine. Kinetic parameters were also obtained for inhibition of 5′-ddThd influx by IdUrd and for inhibition of dThd influx by 5′-ddThd or 5′-N_3-5′-ddThd (data not shown). These are summarized in Table 3. Permeants of the nucleoside transporter were found to inhibit the influx of both 5′-modified nucleoside analogs in a competitive manner, with K_i values similar to their influx K_m values at 20° (27, 28). Both permeants had similar K_m values for influx via the nucleoside transporter, and these matched their K_i values as competitive inhibitors of dThd influx. The V_{max} value for 5′-ddThd was 4-fold higher than that of 5′-N_3-5′-ddThd. The nonfacilitated rate constant for 5′-N_3-5′-ddThd was almost 2-fold higher than that of 5′-ddThd.

Discussion

The papaverine-stop assay was effective for measurement of the initial velocity of influx of 5′-ddThd and 5′-N_3-5′-ddThd into human erythrocytes when the cells were centrifuged within 15 sec of the addition of the cold papaverine solution (Fig. 3; Table 1). Inhibition of influx via both the nucleobase transporter (18, 20, 29) and the nucleoside transporter (20) is attributed to the high concentration of papaverine, and inhibition of nonfacilitated diffusion is attributed to the 7 volumes of cold buffer (both lowering the temperature and diluting the extracellular permeant concentration).

Uptake of both permeants was 1) nonconcentrative, 2) linear with time to at least 8.0 sec, 3) equilibrative across the membrane by 9 min (at 20°), and 4) partially inhibited by nucleosides or inhibitors of nucleoside transport. There was no evidence of metabolism of the permeants.

The influx rates for both permeants were dependent on concentration, with a biphasic profile (Fig. 4), and these data were a good fit to a two-component transport model consisting of a single saturable (carrier-mediated) and linear (nonfacilitated diffusion) entry mechanism. Carrier-mediated influx of both permeants was competitively inhibited by known permeants of the nucleoside transporter (Fig. 5), and both permeants were competitive inhibitors of dThd influx (Table 3), with K_i values being equal to K_m values for influx. We, therefore, conclude that 5′-ddThd and 5′-N_3-5′-ddThd enter human erythrocytes both via the nucleoside transporter and by nonfacilitated diffusion, with kinetic parameters as summarized in Table 3.

At 1.0 μM permeant concentration, the degree of inhibition by nucleosides or inhibitors of nucleoside transport (Table 2) was greater for 5′-ddThd influx (≤94% inhibition) than for 5′-N_3-5′-ddThd influx (≤60% inhibition). The profiles for the concentration dependence of influx rates for these two permeants were also quantitatively different (Fig. 4). These differences reflect both the higher V_{max} value for the nucleoside carrier for 5′-ddThd and the higher nonfacilitated rate constant for 5′-N_3-5′-ddThd.

Whereas both 3′-ddThd and 3′-N_3-3′-ddThd were shown to inhibit dThd influx via the nucleoside transporter (1, 2), no evidence of carrier-mediated transport of these compounds was detected. In contrast, 5′-ddThd and 5′-N_3-5′-ddThd, although exhibiting a 3-fold decreased affinity for the nucleoside transporter, relative to dThd, were permeants for this carrier. Re-

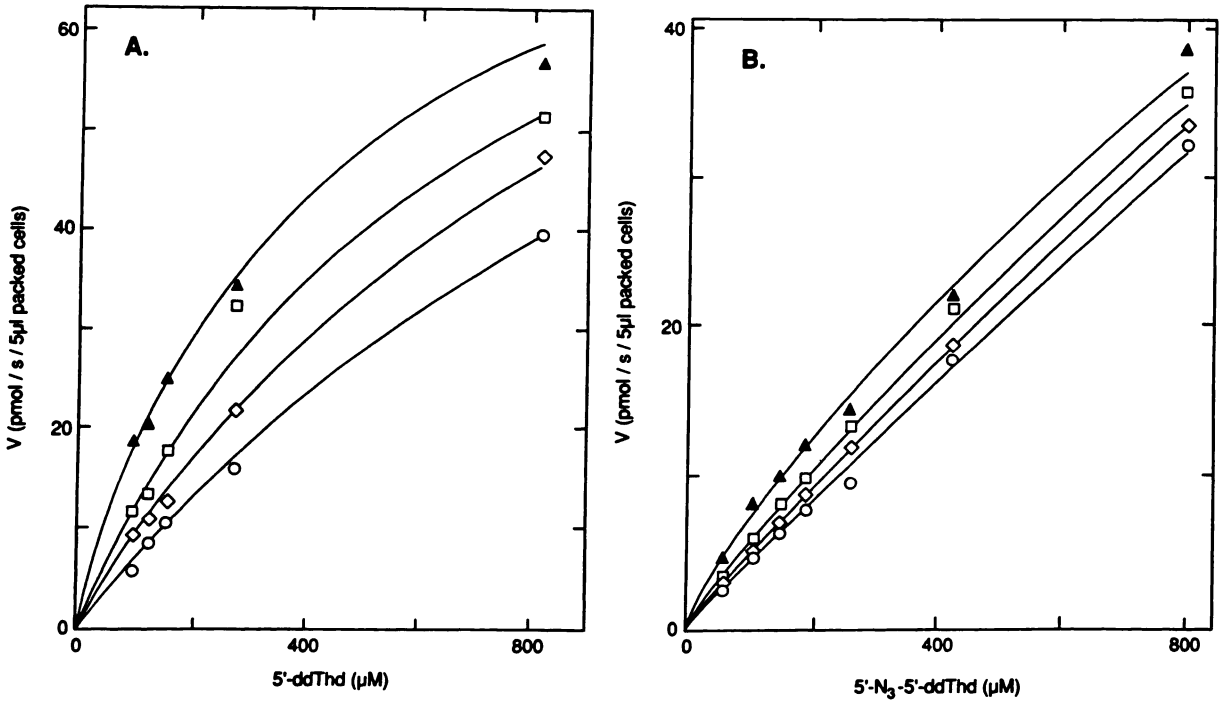

Fig. 5. Analysis of the inhibition of the influx of 5′-ddThd by dThd (A) and that of 5′-N_3-5′-ddThd by 2-chloroadenosine (B). Influx assays were performed at 20°, as described in Experimental Procedures, and velocities were derived from linear regression analysis of data obtained during the linear phase of influx, at 0, 0.5, 1.0, and 2.0 sec (A) and 0, 2.0, 4.0, 6.0, and 8.0 sec (B). Curves were derived by nonlinear regression analysis for competitive inhibition, using eq. 2, given in Experimental Procedures, for [^{3}H]5′-ddThd (2.8 Ci/mol) influx in the absence (▲) or presence of 70 (□), 140 (◇), or 280 (○) μM dThd (A) and for [^{3}H]5′-N_3-5′-ddThd (7.3 Ci/mol) influx in the absence (▲) or presence of 22.5 (□), 45.0 (◇), or 90.0 (○) μM 2-chloroadenosine (B).

TABLE 3

Kinetic parameters for the influx into human erythrocytes of 5′-ddThd and 5′-N_3-5′-ddThd

The initial velocity of influx of each radiolabeled permeant was determined at 20° as described in Experimental Procedures. Inhibitors were added simultaneously with the permeants, and initial velocities were derived by linear regression analysis of the slopes of plots of cell-associated radioactivity versus assay time, using data obtained during the linear phase (0, 0.5, 1.0, and 2.0 sec for 5′-ddThd influx; 0, 2.0, 4.0, 6.0, and 8.0 sec for 5′-N_3-5′-ddThd influx; and 0, 0.4, 0.4, 0.8, and 0.8 sec for dThd influx). Nonlinear regression analysis was performed as described in Experimental Procedures, using Eqs. 2 and 3.

	Permeant influx kinetic parameters			K_i				
	K_m	V_{max}	c^a	dThd	IdUrd	2-Chloroadenosine	5′-ddThd	5′-N_3-5′-ddThd
	μM	pmol/sec/5 μl of cells	fmol/sec/μM/5 μl of cells	μM				
5′-ddThd	200 ± 30[b]	52 ± 3[b]	20 ± 2[b]	86 ± 22[c] (0.999)[d]	84 ± 10[c] (0.20)[d]	ND[e]	ND	ND
5′-N_3-5′-ddThd	220 ± 20[b]	14 ± 1[b]	36 ± 3[b]	ND	ND	18 ± 4[c] (1.0)[f]	ND	ND
dThd	60 ± 1[b]	130 ± 20[b]	<0.2[g]	ND	ND	ND	150 ± 20[h]	200 ± 10[h]

[a] Nonfacilitated diffusion rate constant.
[b] Value is the mean of two determinations ± the average deviation from the mean.
[c] The K_i value ± standard error of that value for the fit of 20 data points to eq. 2 for competitive inhibition.
[d] p value for competitive versus noncompetitive inhibition.
[e] ND, not determined.
[f] The K_i value ± standard error of that value for the fit of 28 data points to eq. 2 for competitive inhibition.
[g] This upper limit for the rate of nonfacilitated diffusion of dThd is based upon an estimation of the residual rate of influx of 1.0 mM dThd into human erythrocytes, at 20°, in the presence of either 4.0 μM NBMPR or 10 μM dilazep; both inhibitors reduced the rate of dThd influx by >99.8%.
[h] The K_i value ± standard error of that value for the fit of 20 data points to the competitive inhibition model (37).

moval of the 5′-OH group of dThd (5′-ddThd) resulted in a 2-fold lowering of the V_{max} for the nucleoside transporter. 5′-ddThd also differed from dThd by exhibiting a significant degree of membrane permeation by nonfacilitated diffusion. The addition of the 5′-azido moiety to 5′-ddThd further reduced the V_{max} for carrier-mediated influx and doubled the rate constant for nonfacilitated diffusion. Two other 5′-modified nucleoside analogs, 5′-deoxyadenosine (30) and 5′-deoxy-5′-methylthioadenosine (31), have been reported to be permeants of the nucleoside transporter, also with diminished affinities, as well as to permeate cells by nonfacilitated diffusion. The importance of the 3′-OH moiety for recognition by the nucleoside transporter has been demonstrated previously (32, 33). It appears that the 5′-OH group may also play a role in permeation efficiency, although apparently not as important a one as that of the 3′-OH group.

The contrast in the nonfacilitated diffusion characteristics of these 3′- and 5′-modified dThd analogs is striking. Whereas the addition of the 5′-N_3- group to 5′-ddThd enhanced the rate constant by only a factor of 2, addition of the 3′-N_3- group to 3′-ddThd enhanced the rate constant by a factor of 4. These enhancements in nonfacilitated diffusion can be correlated with corresponding increases in the octanol/buffer partition coefficients (2). However, the partition coefficients for 5′-N_3-5′-ddThd and 3′-N_3-3′-ddThd are similar; yet the nonfacilitated diffusion rate constants for these analogs differ by 3-fold. Conversely, the partition coefficients for 3′-ddThd and 5′-ddThd differ by a factor of 3, yet the nonfacilitated diffusion rate constants for these analogs are similar. This suggests that the 3′- and 5′-hydroxyl moieties of dThd confer different physicochemical properties not reflected by the octanol/buffer partition coefficients, which affect the ability of the molecule to permeate cells by nonfacilitated diffusion. The role of desolvation energy in determining the rates of nonfacilitated diffusion of 5′-N_3-5′-ddThd and 3′-N_3-3′-ddThd has been described previously (34). This parameter has also been postulated as a determinant in the differences in nonfacilitated diffusion observed with acyclovir and desciclovir (35). A model system has been developed to examine the role of desolvation energies in the nonfacilitated diffusion of the four dThd analogs described in this report, and this is presented in the companion paper (36).

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Send reprint requests to: Dr. Barbara A. Domin, Division of Experimental Therapy, Burroughs Wellcome Company, 3030 Cornwallis Road, Research Triangle Park, NC 27709.