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3′-Deoxythymidin-2′-ene Permeation of Human Lymphocyte H9 Cells by Nonfacilitated Diffusion

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SUMMARY

3′-Deoxythymidin-2′-ene (d4T) is a potent and selective inhibitor of human immunodeficiency virus replication in a variety of human cell types and is currently undergoing phase I clinical trials for the treatment of acquired immunodeficiency syndrome. As part of our ongoing studies of the cellular pharmacology of d4T, and in light of recent reports in which such nucleoside analogs as 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyadenosine were shown to permeate cells by the unusual mechanism of nonfacilitated diffusion, we have investigated the uptake of d4T in the human lymphocyte cell line H9. Several lines of evidence suggest that d4T permeation of H9 cells occurs by nonfacilitated diffusion; 1) [^{3}H]d4T influx was linear for the first 10 sec and was nonconcentrative, reaching equilibrium with the extracellular drug concentration in 2–3 min, 2) the initial rates of influx were a linear function of concentration over the range from 1 μM to 5 mM, with no sign of uptake by a saturable mechanism, and 3) the uptake of [^{3}H]d4T was insensitive to the nucleoside transport inhibitors nitrobenzylthioinosine and dipyridamole, as well as a large molar excess of AZT, thymidine, or adenosine. The octanol/water partition coefficient of d4T was 0.179, intermediate between those of thymidine and AZT. Thus, d4T does not appear to be a substrate for the nucleoside transport system responsible for the uptake of physiological nucleosides as well as most nucleoside analogs, and it enters the cell by nonfacilitated diffusion.

Physiological nucleosides, as well as most nucleoside analogs, permeate mammalian cells by utilizing one or more of several carrier proteins that possess broad specificities (1–3). Recent evidence, however, has shown that some nucleoside analogs, among them several of those shown to be potent inhibitors of the replication of HIV-1, permeate the cell membrane independent of this carrier, by nonfacilitated diffusion. These include AZT (4), 2′,3′-dideoxyadenosine (5, 6), and d2T (7). 2′,3′-Dideoxycytidine permeation of human erythrocytes has recently been shown to be partly, although very inefficiently, mediated by a nucleoside carrier (8). This unique property has been attributed to an increase in lipophilicity relative to the parent nucleoside (4), although it has also been observed that the murine erythrocyte nucleoside transporter is sensitive to substrate alterations at the 3′-position (9), a characteristic that these compounds share.

d4T has been shown by this laboratory and others to be a potent and selective inhibitor of HIV-1 replication and of cytopathic effects in a variety of cell types (10–12). We have shown that d4T is relatively nontoxic to the uninfected human T cell line H9, is phosphorylated by cellular kinases to the mono-, di-, and triphosphate level, and is incorporated ultimately into the DNA of growing cells (13). Similar results on the metabolism of d4T have been reported in MT-4 and CEM cells (14, 15). The 5′-triphosphate of d4T has been shown to be a potent inhibitor of HIV-1 reverse transcriptase (16). d4T is currently undergoing phase I clinical trials for the treatment of acquired immunodeficiency syndrome (17).

In light of the potent and selective antiviral activity of d4T against HIV-1 and as a part of our ongoing studies of the cellular pharmacology of d4T, we have investigated the mechanism of uptake of d4T in the human lymphocyte cell line H9. Our results show that the uptake of d4T in H9 cells is not carrier mediated and occurs by nonfacilitated diffusion. Moreover, d4T is not a substrate for the nucleoside transporter responsible for the uptake of physiological nucleosides. A preliminary account of this research has been presented.[1]

Materials and Methods

Chemicals. [*methyl*-^{3}H]d4T (20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Nonradioactive d4T was synthesized

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ABBREVIATIONS: HIV-1, human immunodeficiency virus 1; d4T, 3′-deoxythymidin-2′-ene; AZT, 3′-azido-3′-deoxythymidine; d2T, 3′-deoxythymidine; dThd, thymidine; NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

by the procedure of Lin *et al.* (10), and AZT was synthesized according to the method of Lin and Prusoff (18). [*methyl*-^{3}H]dThd (60 Ci/mmol) was obtained from ICN Biomedicals (Costa Mesa, CA). NBMPR, dipyridamole, dThd, and adenine were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. H9 cells were maintained in RPMI 1640 supplemented with 1% each of heat-inactivated fetal bovine and newborn calf serum and HL-1 serum substitute (Ventrex Laboratories, Portland, ME), plus 10 mM HEPES, pH 7.4, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37° in a humidified 5% CO_2 atmosphere. Under these conditions, the cells doubled every 24 hr.

Uptake studies. Uptake of radiolabeled permeant (d4T or dThd) was determined by an "oil stop" methodology (19) with slight modification. Oil stop tubes were prepared by layering 100 μl of a silicone/paraffin oil mixture (Dow Corning DC550 silicone oil/Fischer light paraffin oil, 78:22, v/v) over 50 μl of 7% $HClO_4$ in a 400-μl microcentrifuge tube. Above the oil layer were placed 100 μl of an assay mixture containing the radiolabeled permeant in medium at twice the desired final concentration. H9 cells were suspended in medium at 1×10^7 cells/ml and kept in a 37° water bath. The prepared oil stop tubes were balanced in an Eppendorf model 5412 microcentrifuge; at intervals timed audibly by a metronome, 100 μl of the cell suspension, containing 1×10^6 cells, were forcibly dispensed into each assay tube from a repeating syringe, and the cells were then separated from the incubation medium by centrifugation at 15,000 × *g* for 30 sec. The tubes were rapidly frozen in dry ice and cut through the oil layer, and each portion was placed in a plastic scintillation vial, containing 0.2 ml of H_2O, and vortexed vigorously. The radioactivity in each portion was determined in 5 ml of Optifluor (Packard Instrument Co., Downers Grove, IL).

Uptake during extended time courses (i.e., time points of >1 min) was determined by the technique described above, except that cells and radioactive permeant were incubated together in medium at 37°. A 200-μl aliquot of cells and permeant was layered over the prepared oil stop tube at the desired time points and processed for uptake as described above.

In separate determinations, the total and extracellular water spaces of the cell pellet were measured by incubation of cells with ^3H_2O (5.5×10^3 dpm/μl) and [^{14}C]inulin (1.1×10^3 dpm/μl) for approximately 5 min, centrifugation as described above, and determination of the dpm for each isotope in the cell pellet. The intracellular volume of the cell pellet (μl of cell H_2O) was defined as [(^{3}H-related) − (^{14}C-related)] dpm and the extracellular H_2O space or volume of trapped medium as ^{14}C-related dpm. These values were 2.13 ± 0.30 and 0.53 ± 0.04 μl (nine experiments), respectively. Drug uptake data were corrected for extracellular radioactivity present in the pellet; no attempt was made to correct for the lag time required for effective separation of the cells from the extracellular permeant after activation of the microcentrifuge. Initial velocities were determined by linear regression analysis of the slopes of plots of cell-associated [^{3}H]d4T or [^{3}H]dThd versus time, using data obtained during the linear phase (3–9 sec) of influx.

Metabolic inertness of d4T. H9 cells were incubated for 10 sec or 1 min with 500 μM [^{3}H]d4T and were separated from the incubation medium as described above. The $HClO_4$-soluble fractions from triplicate oil stop tubes at each time point were pooled, neutralized with KOH, and analyzed by reverse phase high pressure liquid chromatography. The high pressure liquid chromatographic analyses were performed on an 8-mm × 10 cm μBondapak cartridge column (Waters), employing a linear gradient of 0–20% CH_3CN in 0.1 M $(NH_4)OOCCH_3$, pH 5.5, over 40 min at 3 ml/min.

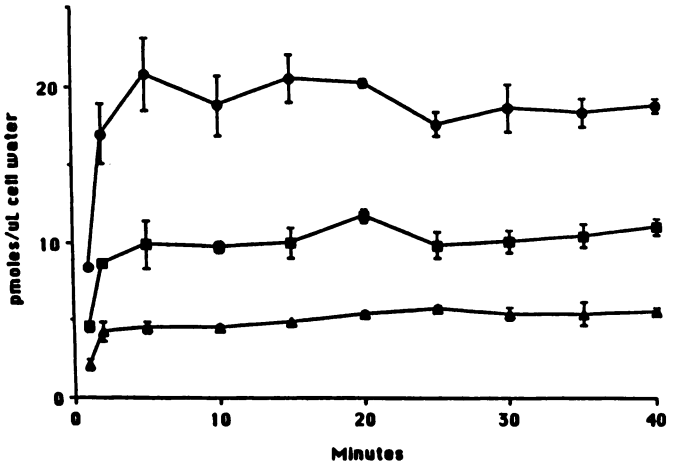

Fig. 1. Time dependence of influx of [^{3}H]d4T (500 cpm/pmol) into H9 cells. ▲, 5 μM; ■, 10 μM; ●, 20 μM [^{3}H]d4T. Uptake was determined as described in Materials and Methods, and each *point* reflects the mean ± standard deviation of at least triplicate determinations.

Results

Time dependence of uptake of d4T. The time dependence of influx of 5, 10, and 20 μM [^{3}H]d4T into H9 cells, a human T lymphoid cell line, is shown in Fig. 1. The influx was linear for approximately the first 10 sec and reached equilibrium with the

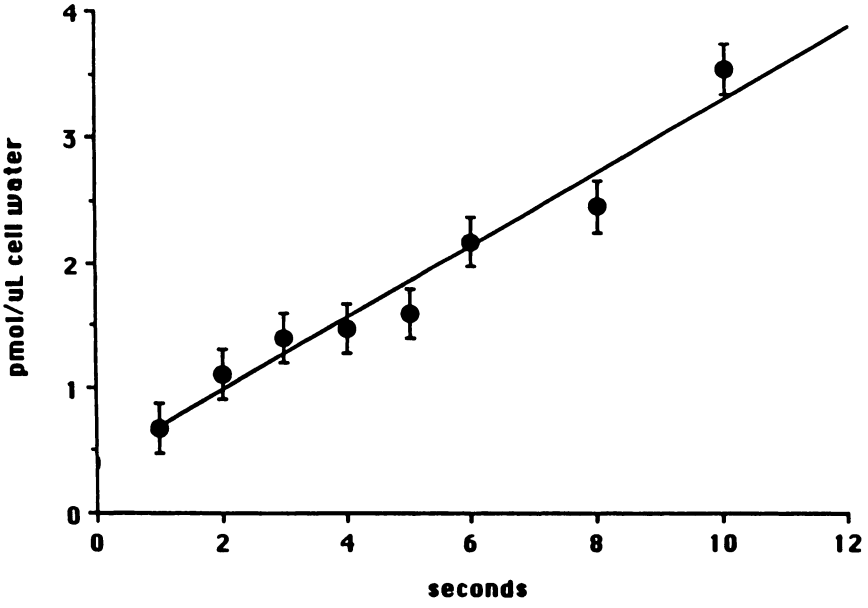

Fig. 2. Initial rate determination of the influx of 20 μM [^{3}H]d4T. Each *point* reflects the mean ± standard deviation of at least triplicate determinations, and the slope corresponds to an initial rate of 0.30 pmol/μl/sec (r = 0.98).

extracellular drug concentration within approximately 2 min. Intracellular [^{3}H]d4T remained in stable equilibrium with the extracellular concentration for at least 40 min and, thus, the uptake of d4T is nonconcentrative.

To further characterize the initial rate of d4T uptake, the influx of 20 μM [^{3}H]d4T was measured during the first 10 sec of exposure (Fig. 2). In agreement with Fig. 1, influx was linear for at least the first 10 sec and corresponded to an initial rate of 0.30 pmol/μl/sec at this concentration.

In separate experiments, we observed no phosphorylation of even high concentrations of d4T by H9 cells during the first 1 min of incubation (more than 99% of the cell-associated radioactivity coeluted with authentic d4T at all time points) and, thus, the uptake determined during the initial rate period (1–10 sec) reflects true influx of d4T and not a composite of uptake and metabolism.

Concentration dependence of initial rates. The initial rates of uptake of [^{3}H]d4T were determined over a range of concentrations from 1 μM to 5 mM. As shown in Fig. 3, no saturation of initial rate was observed over this 5000-fold change in extracellular d4T concentration, suggesting that d4T uptake does not occur by a carrier-mediated process.

Effect of inhibitors on d4T and dThd uptake. We next determined the effect of two inhibitors of nucleoside transport, as well as exogenous nucleosides and a nucleobase, on the uptake of [^{3}H]d4T and [^{3}H]dThd by H9 cells. As shown in Table 1, the initial rate of d4T influx (0.0039 pmol/μl/sec) was some 10-fold slower than that of dThd influx (0.044 pmol/μl/sec). Under conditions where the carrier-mediated uptake of 1 μM [^{3}H]dThd was 86 and 94% inhibited by NBMPR and dipyridamole, respectively, the uptake of 1 μM [^{3}H]d4T was unaffected (no significant difference in rates versus control, as analyzed by Student's t test, $p > 0.05$). Likewise, the presence of a 1000-fold molar excess of unlabeled dThd, AZT, or d4T had little or no effect on the uptake of [^{3}H]d4T ($p > 0.05$, Student's t test). Finally, the presence of a 1000-fold molar excess of adenine was ineffective in inhibiting d4T uptake.

Octanol/H_2O partition coefficients. The partition coefficient in n-octanol/10 mM phosphate buffer (pH 7.4) of d4T was compared with that of dThd, d2T, and AZT (Table 2). The values of 0.085, 0.250, and 0.983 for dThd, d2T, and AZT, respectively, are in agreement with those reported previously (7). Although d4T, with a partition coefficient of 0.179, is more hydrophobic than dThd, both d4T and d2T are considerably less hydrophobic than AZT.

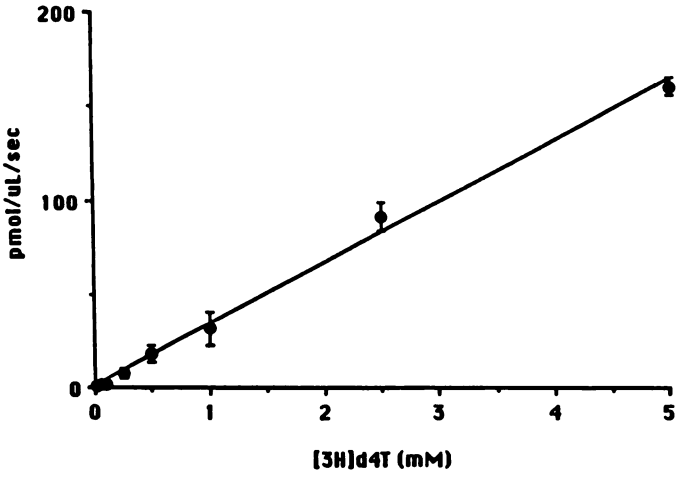

Fig. 3. Concentration dependence of initial rate of influx of [^{3}H]d4T. Initial rates were determined as described in Materials and Methods, from time courses similar to that of Fig. 2, during the first 3–9 sec of influx. Each *point* represents the mean ± standard deviation of at least triplicate time courses.

TABLE 1

Effect of various transport inhibitors and nucleosides on the initial rate of uptake of [^{3}H]d4T and [^{3}H]dThd

H9 cells were preincubated with NBMPR or dipyridamole for 15 min at 37° before determination of the initial rate of influx of [^{3}H]d4T or [^{3}H]dThd (3200 cpm/pmol). In the remaining experiments, the competing nucleosides were incorporated into the assay mixture and introduced along with the radiolabeled permeant. Initial rates were determined as described in Materials and Methods. Each value represents the mean ± standard deviation of at least triplicate determinations.

	Initial rate	
	[^{3}H]d4T (1 μM)[a]	[^{3}H]dThd (1 μM)
	pmol/μl/sec	
Control	0.0039 ± 0.0006	0.044 ± 0.002
+20 μM NBMPR	0.0037 ± 0.0003	0.006 ± 0.003
+20 μM Dipyridamole	0.0030 ± 0.0006	0.007 ± 0.002
+1 mM dThd	0.0034 ± 0.0008	ND[b]
+1 mM AZT	0.0039 ± 0.0001	ND
+1 mM d4T	0.0035 ± 0.0007[c]	ND
+1 mM Adenine	0.0032 ± 0.0009	ND

[a] As discussed in the text, the rates of [^{3}H]d4T uptake were judged not to be significantly different from control ($p > 0.05$).

[b] ND, not determined.

[c] This value represents the influx rate of radioactivity from 1 μM [^{3}H]d4T without correction for change in specific activity resulting from the addition of nonradiolabeled d4T as an inhibitor.

TABLE 2

Octanol/H_2O partition coefficients of thymidine analogs

Partition coefficients in n-octanol/10 mM potassium phosphate buffer (pH 7.4) were determined by the procedure of Lin (23). Each value represents the mean ± standard deviation of at least duplicate determinations.

Compound	Partition coefficient
dThd	0.085 ± 0.002
AZT	0.983 ± 0.056
d2T	0.250 ± 0.003
d4T	0.179 ± 0.001

Discussion

We have presented several lines of evidence to support the conclusion that d4T permeation of human lymphocytes occurs by nonfacilitated diffusion. The initial rate of d4T influx was directly proportional to the extracellular d4T concentration, with no evidence of saturation over a 5000-fold concentration range (Fig. 3). Two potent inhibitors of nucleoside transport (NBMPR and dipyridamole) were unable to alter the initial rate of d4T influx (Table 1). These agents bind with high affinity to the nucleoside transport protein and block binding and translocation of a nucleoside substrate (1, 2). A 1000-fold molar excess of dThd, known to be a substrate for the nucleoside transporter, had no effect on the initial rate of d4T influx (Table 1). Likewise adenine, which enters cells via a distinct nucleobase carrier (1), did not inhibit d4T influx (Table 1). Acyclovir, an acyclic deoxyguanosine analog, has recently been shown to also utilize this nucleobase carrier (20). Finally, a 1000-fold molar excess of unlabeled d4T was unable to inhibit the uptake of 1 μM [^{3}H]d4T. Similar results were obtained in studies of AZT and d2T uptake (4, 7). Thus, our results demonstrate that d4T is not a substrate for the nucleoside transport system, which mediates the entry of physiological nucleosides

and most nucleoside analogs studied to date (2, 3), and that d4T enters the cell chiefly via nonfacilitated diffusion.

Recently, it has been shown that various other 3′-deoxy-3′-substituted nucleoside analogs also bypass the nucleoside carrier, including AZT (4), d2T (7), and 2′,3′-dideoxyadenosine (5, 6). These compounds all share the characteristics of lacking the 3′-hydroxyl and possessing greater lipophilic character, relative to the natural parent nucleoside. For example, d2T has a partition coefficient over twice that of dThd (Table 2 and Ref. 7).

The substitution of an azido group for the 3′-hydroxyl of dThd results in an increase of >10-fold in the partition coefficient (Table 2 and Ref. 4). Although d4T is less lipophilic than its 2′,3′ saturated counterpart (d2T), it nonetheless is more lipophilic than dThd. Zimmerman *et al.* (4) speculated that the increase in lipophilicity of AZT over dThd is responsible for the permeation of cells by AZT by nonfacilitated diffusion. The rapid, non-carrier-mediated permeation of human erythrocytes by 2′,3′-dideoxyadenosine has also been attributed to its lipid solubility [partition coefficient = 0.609 (5)]. However, Gati *et al.* (9) have shown that modification at the 3′-position of a nucleoside confers altered affinity for the mouse erythrocyte nucleoside transporter. In the case of d4T, it seems likely that both factors operate to allow the compound to circumvent the nucleoside transporter; the lack of a 3′-OH results in decreased affinity for the carrier (relative to dThd), and the relative lipid solubility enables d4T to permeate the cell membrane by simple diffusion. Zimmerman *et al.* (4) reported an initial rate of 0.0223 pmol/μl/sec for the influx of 1 μM [^{3}H]AZT (4). The initial rate of influx of 1 μM [^{3}H]d4T reported here (0.0039 pmol/μl/sec; Table 1) is over 5-fold slower than that of AZT, which is consistent with a difference in lipophilic character of the two compounds (Table 2).

Although d4T uptake is slow, compared with that of dThd and AZT (Table 1), presumably due to the lack of affinity of d4T for the nucleoside transporter and its comparative hydrophilicity (relative to AZT), which limits the rate of passive diffusion through the membrane, the permeation of d4T through the cell membrane does not appear to be rate limiting in its metabolism. We have previously reported a significant pool of unphosphorylated d4T present in H9 cells, as well as low but stable levels of the triphosphate (13). In addition, d4T is a poor substrate for purified cytosolic thymidine kinase [K_m = 138 μM (21)]. Thus, it is the initial phosphorylation of d4T to its monophosphate that is the rate-limiting step in its metabolism.

The mechanism by which a drug traverses the cell membrane may have profound effects on its bioavailability, distribution, and pharmacokinetics. The favorable pharmacokinetics and central nervous system penetration of AZT are consistent with passage across cell membranes by simple diffusion, allowing access to every compartment of the body (4). In studies comparing d4T and AZT in mice, Russell *et al.* (22) have reported similar pharmacokinetic characteristics, including rapid absorption, complete biovabilability, and significant penetration into the central nervous system. Thus, these data also support our conclusion that d4T entry into cells occurs via simple non-mediated diffusion.

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