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PERMEATION AND METABOLISM OF ANTI-HIV AND ENDOGENOUS NUCLEOSIDES IN HUMAN IMMUNE EFFECTOR CELLS

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Abstract—Numerous anti-HIV drugs are synthetic analogs of endogenous nucleosides. Therefore it is of interest to see if a facilitated nucleoside transport system exists to mediate their uptake into human immune effector cells that are known HIV targets. Nucleoside permeation and metabolism in lymphocytes, macrophages and bone marrow cells isolated from healthy human volunteers were studied, using uridine as the prototype endogenous nucleoside. There are saturable broad specificity nucleoside transport systems in all three cell types, all of which were inhibited by dipyridamole. The V_{max} and K_m values for uridine transport were 0.05 ± 0.01 pmol/sec/10^6 cells and 18.4 ± 4.2 μM, respectively, for lymphocytes, 0.04 ± 0.01 pmol/sec/10^6 cells and 25.3 ± 6.6 μM, respectively, for macrophages, and 0.03 ± 0.01 pmol/sec/10^6 cells and 90.2 ± 10.1 μM, respectively, for bone marrow mononuclear cells. Anti-HIV dideoxynucleosides such as azidothymidine (AZT), 2′,3′-dideoxycytidine (DDC), 2′,3′-dideoxyinosine (DDI), 2′,3′-dideoxyadenosine (DDA), and 2′,3′-dideoxythymidine (DDT) are not substrates of this nucleoside transport system; hence, little or no drug accumulated inside the cells after 60 sec. Equilibration of cells with uridine or dideoxynucleosides for 2 hr resulted in high levels of cellular uridine and DDA, low levels of cellular AZT, but undetectable levels of the other analogs in all three cell types. Active metabolite levels in lymphocytes as assayed by HPLC correlated with the drug permeation results. Our data demonstrated that DDC, DDI, and DDT are not substrates for the nucleoside transporter and cannot diffuse readily across the cell membrane of human immune effector cells. Future anti-HIV drug development efforts should consider drugs that are substrates of the nucleoside transporter to ensure rapid and complete uptake into target cells.

Many synthetic dideoxynucleosides and related compounds are currently undergoing preclinical and clinical testing for their ability to inhibit HIV replication [1–3]. These anti-HIV compounds are analogs of endogenous nucleosides that are normally present in the body as metabolic products of the nucleic acid biosynthetic pathways. There is a broad specificity nucleoside transporter, a component of salvage metabolism, on mammalian cell membranes, which is responsible for the non-concentrative facilitated uptake of endogenous nucleosides into many cells [4–6]. Lymphocytes, macrophages and bone marrow cells are reported HIV targets or sanctuaries in infected individuals [7–10]. Although a facilitated diffusion process for nucleosides has been characterized in human erythrocytes [6], it is not clear whether human lymphocytes, macrophages, and bone marrow cells have facilitated nucleoside transport systems. Furthermore, the role that salvage metabolism (or a nucleoside transport system) plays in cellular nucleic acid metabolism in these cells is unknown. Although azidothymidine (AZT§) has been found to permeate human red blood cells and lymphocytes by passive diffusion rather than by facilitated mechanisms [11], it is unclear at the moment whether any of the other anti-HIV dideoxynucleosides are, in fact, substrates of a facilitated uptake system. The permeation of dideoxynucleosides across cell membranes, whether by facilitated transport or by passive diffusion, is a crucial step prior to their metabolic activation in target cells. We report here a study examining the permeation and metabolism of uridine (an endogenous nucleoside) and five dideoxynucleosides in lymphocytes, macrophages, and bone marrow mononuclear cells isolated from healthy human volunteers. The data reported here should provide insight into future anti-HIV drug development, and may lead to eventual targeting of drugs toward specific types of immune effector cells in AIDS patients.

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§ Abbreviations: AZT, azidothymidine; DDC, 2′,3′-dideoxycytidine; DDI, 2′,3′-dideoxyinosine; DDA, 2′,3′-dideoxyadenosine; and DDT, 2′,3′-dideoxythymidine.

MATERIALS AND METHODS

Lymphocyte and macrophage isolation from human volunteers. The protocols for blood and bone marrow collection have been approved by the institutional Human Subjects Committee. After obtaining informed consent, peripheral blood was drawn from healthy volunteers via the antecubital vein into vacutainer tubes containing EDTA. A

blood sample from each volunteer was submitted to a clinical laboratory for complete blood count, chemistry panel, serology, HIV and hepatitis screens to document the donor as healthy. Lymphocytes were isolated by mixing each 3 mL of blood with 5 mL of Dulbecco's phosphate-buffered saline (PBS), and the suspension was layered carefully on top of 3 mL of Ficoll-Hypaque (Histopaque 1077, Sigma Chemical Co., St. Louis, MO). The tube was centrifuged at 400 *g* for 30 min, and the cells at the interface were collected. These cells were then washed twice with 10 mL of PBS and centrifuged at 250 *g* for 10 min. The cell pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated at 37° for 2 hr in a humidified incubator under a 5% CO_2 atmosphere in cell culture flasks. The non-adherent cells were 95% lymphocytes, and the adherent cells were mostly macrophages (greater than 90%). After removing the lymphocytes, the macrophages were harvested by trypsin/EDTA treatment. Both cell types were resuspended in RPMI medium with 10% dialyzed fetal bovine serum (Sigma), and the cell density was quantified by electronic (Coulter Counter Zf, Hialeah, FL) or hemocytometer (with trypan blue staining) counting. This isolation procedure typically recovered 70% of the lymphocytes with greater than 98% viability, and macrophages were recovered with greater than 90% viability.

Isolation of bone marrow mononuclear cells. Bone marrow samples (4.5 mL of bone marrow aspirated into a syringe with 0.5 mL of heparinized saline) were aspirated from healthy paid human volunteers at the posterior iliac crest under local anesthesia after obtaining their informed consent as previously described [12]. Blood samples were also submitted to a clinical laboratory to confirm the health of each donor as listed above. The bone marrow cells were mechanically dispersed by expulsion through successively smaller gauge hypodermic needles into α-Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum. The cells were then layered over a Ficoll-Hypaque cushion and centrifuged at 1200 *g* for 30 min. Mononuclear cells collected from the interface were washed twice in PBS and resuspended in α-MEM supplemented with 10% dialyzed fetal bovine serum. Cell counts were established electronically before initiating nucleoside transport studies.

Nucleoside transport measurements. A modified oil-stop procedure was used to measure zero-trans influx (initial uptake of nucleosides before significant accumulation or metabolism inside cells) of uridine and the dideoxynucleoside analogs as described previously [13]. Briefly, 100-μL aliquots of RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and containing radiolabeled nucleosides (1 μM, 5–20 μCi/mL) plus various concentrations of unlabeled nucleosides or dipyridamole (0.01 to 10 μM) were layered carefully onto 100-μL aliquots of an oil mixture of 9:1 silicone oil (Aldrich 17563-3):paraffin oil (Fisher 0-119) in 1.5 mL Eppendorf microcentrifuge tubes. Radiolabeled [2′,3′-^{3}H(*N*)]-2′,3′-dideoxyinosine ([^{3}H]-DDI), 2′,3′-dideoxyadenosine ([^{3}H]DDA), 2′,3′-dideoxythymidine ([^{3}H]DDT), and [5,6-^{3}H]-2′,3′-dideoxycytidine ([^{3}H]DDC) were purchased from Moravek Biochemicals, Brea, CA, and [5,6-^{3}H]-uridine was purchased from Amersham, Arlington Heights, IL. Transport measurements at 25° were initiated by the addition of 100 μL of cell suspension (5×10^5 cells), and the reactions were stopped at timed intervals between 0 and 60 sec by pelleting the cells through the oil cushion at 12,000 *g* for 30 sec in a microcentrifuge. The medium and the oil cushion were aspirated and the cell pellet was washed once with 1 mL of chilled PBS. The cells were digested with 0.1 M NaOH and the radioactivity was quantified using liquid scintillation counting. The typical loss of radioactivity from the wash procedure at 4° was less than 2% [14].

Long-term nucleoside uptake. The long-term uptake rates were estimated from freshly isolated cells suspended in RPMI 1640 medium supplemented with dialyzed serum. Radiolabeled dideoxynucleosides were added to the cell suspensions and incubated at 37°. Aliquots of cells were removed at timed intervals, diluted into 10 vol. of chilled PBS, and centrifuged for 5 min at 1000 *g*. The cell pellets were washed twice with chilled PBS and digested in 0.1 M NaOH; then the radioactivity of the extracts was measured by liquid scintillation counting [15]. The values for bone marrow mononuclear cells represent an average drug concentration in the subpopulations present.

Measurements of cellular nucleotide levels. Human peripheral lymphocytes were incubated for 2 hr in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum with or without rhIL-1 (0.1 μg/mL), washed, and re-incubated for 24 hr in medium containing the radiolabeled nucleoside of interest. Cells were washed twice with chilled PBS, extracted with 200 μL of 0.6 N perchloric acid on ice, and neutralized with 2.2 M KH_2PO_4. Aliquots were then injected into an HPLC system for quantitation of radioactivity in the nucleotides. The HPLC system consisted of two Waters 6000A pumps (Waters, Bedford, MA), a Waters SAX-anion exchange column in a 8 × 10 RCM module, a Waters 490 multiwavelength detector and a Radiomatic A-100 flow scintillation detector with a scintillant (Floscint 3, Radiomatic, Tampa, FL) flow of 3 mL/min. The Waters Maxima 820 HPLC software running on a NEC Powermate II microcomputer was used to control flow rate, gradient profile, and for data acquisition and analysis. The initial conditions were: 80% Buffer A (0.1 M KH_2PO_4/0.1 M KCl, pH 4) and 20% Buffer B (0.25 M KH_2PO_4/0.5 M KCl, pH 5) at a flow rate of 2 mL/min. The nucleotides were eluted with a hyperbolic gradient (Curve C) from 20 to 100% Buffer B over 35 min at a flow rate of 2 mL/min. Both the real-time and total radioactivity for each peak as well as the UV absorbance were recorded. Radioactivity in peaks that co-eluted with authentic (unlabeled) nucleoside triphosphate standards (Sigma Chemicals) as monitored by UV absorbance (254/280 nm) were used for all calculations.

RESULTS

Using an "oil-stop" method for rapid transport

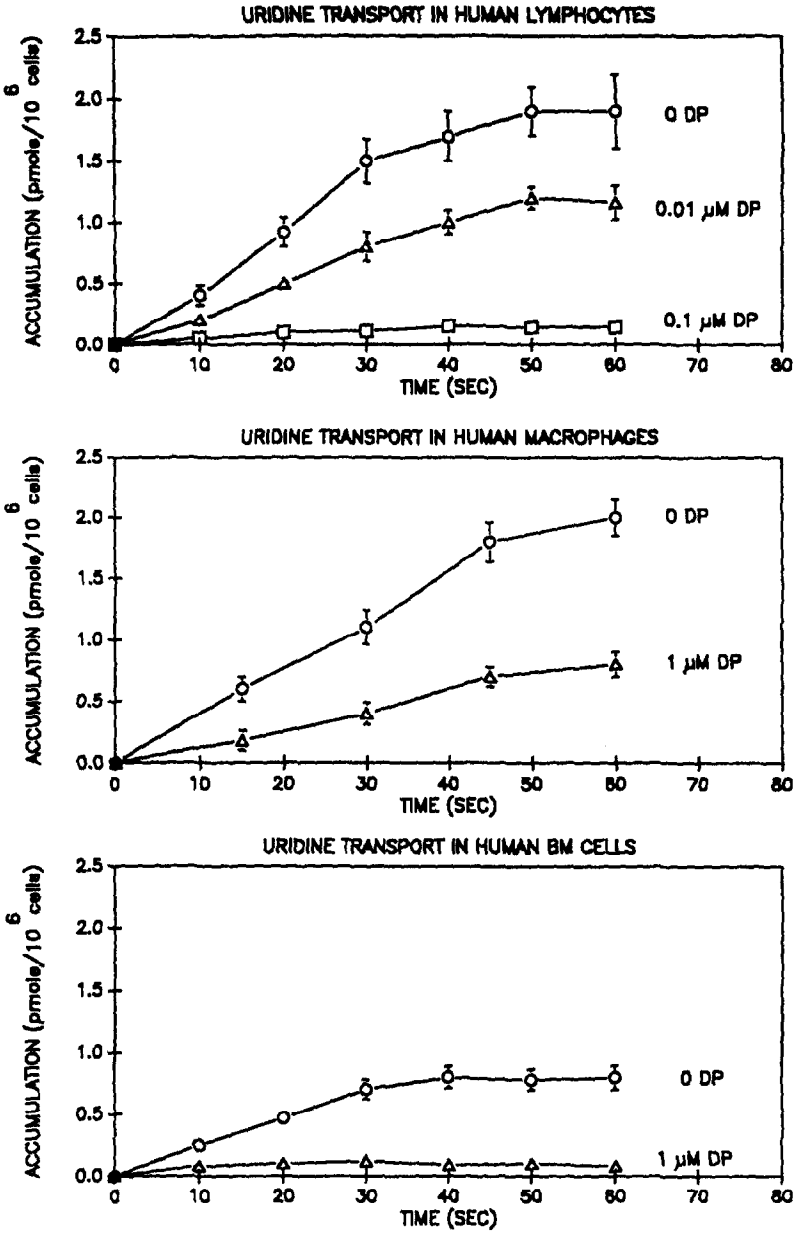

Fig. 1. Zero-trans influx of uridine into human lymphocytes, macrophages, and bone marrow (BM) mononuclear cells in the presence and absence of the nucleoside transport inhibitor dipyridamole (DP). Each point is the mean of four experiments, and the bars denote one standard deviation from the mean.

measurements, we found that human peripheral blood lymphocytes, macrophages, and bone marrow mononuclear cells all exhibited a saturable nucleoside transport system that was inhibitable by dipyridamole (the *es* transporter according to the new nomenclature). Figure 1 shows a comparison of zero-trans influx of [5,6-^{3}H]uridine (the endogenous nucleoside with the highest V_{max} for transport in human erythrocytes) into human lymphocytes, macrophages and bone marrow mononuclear cells. The transport of uridine over the first 40 sec appeared to be rapid and linear in all three cell types; transport leveled off to a slower influx rate beyond this time point. Uridine transport in all three cell types was sensitive to inhibition by dipyridamole, a documented nucleoside transport inhibitor. In the macrophages, the maximum inhibition achievable was 60%, suggesting the presence of a dipyridamole-insensitive uptake system as well. Assuming that Michaelis–Menten kinetics are applicable to our facilitated transport data, the calculated K_m and V_{max} values for the first 40 sec of uridine transport in each cell

Table 1. Parameters of zero-trans uridine influx in different cell types

Cell type	N	V_{max} (pmol/sec/10^6 cells)	K_m (μM)
Lymphocytes	8	0.050 ± 0.011	18.4 ± 4.2
Macrophages	4	0.037 ± 0.014	25.3 ± 6.6
Bone marrow cells	4	0.025 ± 0.007	90.2 ± 10.1

After measuring the zero-trans influx rates of [^{3}H]-uridine under various unlabeled substrate concentrations using the procedure described in Fig. 1, the data were fitted to a curve using an enzyme kinetics curve-fitting algorithm [16] and the parameters were extrapolated. The values reported are the means ± SD of V_{max} and K_m at 25° of the uridine transport process in each human cell type. Since the bone marrow mononuclear cells contain heterogenous subpopulations of cells, the values represent an average of all the cell types present.

type are listed in Table 1. The inhibitory constant (K_i) for dipyridamole in lymphocytes was 0.05 ± 0.02 μM, in macrophages 0.06 ± 0.01 μM, and in bone marrow mononuclear cells 0.15 ± 0.03 μM. The values reported here for bone marrow mononuclear cells represent averages over the subpopulations of cells present.

We also studied the mode of permeation of five dideoxynucleosides into lymphocytes, macrophages and bone marrow mononuclear cells. Table 2 summarizes both the net cellular dideoxynucleoside accumulation after 60 sec (zero-trans influx) as well as their long-term uptake after 2 hr (zero-trans influx plus diffusion plus cellular metabolites) in each of the three cell types. None of the five dideoxynucleosides tested was a substrate of the nucleoside transporter; hence, little or no accumulations were observed in the three cell types after 60 sec when compared to the rapid uridine accumulation. In addition, the small amounts of influx observed for AZT and DDA were slow and not sensitive to inhibition by dipyridamole, suggesting passive diffusion as the mechanism of permeation.

Longer term drug uptake studies were also performed to examine the net cellular accumulation after 2 hr of equilibration. As illustrated in Table 2, uridine and DDA demonstrated fairly high accumulation within this time frame in lymphocytes and bone marrow cells and moderate accumulation in macrophages. AZT accumulations were detectable but remained relatively low in all three cell types. More importantly, DDC, DDI and DDT did not accumulate in any of the three cell types even after such an extended equilibration.

To investigate whether the cellular levels of active metabolites correlated with nucleoside permeation, we measured the concentrations of the corresponding triphosphates for each nucleoside analog in the perchloric acid extracts of lymphocytes after 24 hr of equilibration. Table 3 summarizes our results, which indicate that uridine 5′-triphosphate (retention time = 18 min) was detected at high levels, 2′,3′-dideoxyadenosine 5′-triphosphate (retention time = 25 min) was detected at moderate levels, and azidothymidine 5′-triphosphate (retention time = 21 min) was found at rather low levels. As expected from the zero-trans and long-term uptake data, no 2′,3′-dideoxyinosine 5′-triphosphate (nor its expected metabolite 2′,3′-dideoxyadenosine 5′-triphosphate), 2′,3′-dideoxycytidine 5′-triphosphate, or 2′,3′-dideoxythymidine 5′-triphosphate were detectable in the perchloric acid extract of the lymphocytes. Recombinant human interleukin-1 was used to activate the lymphocytes in a series of experiments and the amounts of cellular radiolabeled triphosphate were not different from unstimulated cells.

Table 2. Net cellular accumulation of dideoxynucleosides

	Cellular accumulation* (pmol/million cells)					
	Lymphocytes		Macrophages		Bone marrow	
	0 DP	1 μM DP	0 DP	1 μM DP	0 DP	1 μM DP
60 Sec						
Uridine	1.8 ± 0.3	0.2 ± 0.1	1.9 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.1 ± 0.1
AZT	0.4 ± 0.2	0.5 ± 0.3	0.6 ± 0.2	0.5 ± 0.3	0.4 ± 0.2	0.3 ± 0.2
DDA	0.9 ± 0.3	1.1 ± 0.4	0.8 ± 0.3	0.9 ± 0.4	0.7 ± 0.1	0.6 ± 0.2
DDC	—	—	—	—	—	—
DDI	—	—	—	—	—	—
DDT	—	—	—	—	—	—
2 Hr						
Uridine	2.6 ± 0.5	0.3 ± 0.2	1.9 ± 0.3	0.2 ± 0.1	6.8 ± 1.2	0.5 ± 0.3
AZT	0.9 ± 0.4	0.7 ± 0.5	0.6 ± 0.3	0.8 ± 0.2	0.9 ± 0.4	1.1 ± 0.5
DDA	2.9 ± 0.4	2.2 ± 0.3	1.3 ± 0.3	1.5 ± 0.4	3.3 ± 0.8	2.9 ± 0.6
DDC	—	—	—	—	—	—
DDI	—	—	—	—	—	—
DDT	—	—	—	—	—	—

* Values are means ± SD of four experiments, and "—" denotes undetectable levels. Net cellular accumulations were calculated from the net radioactivity in cells at 60 sec and 2 hr. 0 DP and 1 μM DP denote the absence and presence of 1 μM dipyridamole, respectively.

Table 3. Human lymphocyte nucleoside triphosphates after 24-hr incubation

	Concentrations in cell extract* (pmol/million cells)					
	No rhIL-1			0.1 μg/mL rhIL-1		
Nucleotide	N	0 DP	1 μM DP	N	0 DP	1 μM DP
UTP	4	4.97 ± 1.12	0.16 ± 0.07	3	5.15 ± 1.06	0.22 ± 0.09
AZTTP	3	0.02 ± 0.01	0.03 ± 0.01	3	0.03 ± 0.02	0.04 ± 0.01
DDATP	2	1.17 ± 0.65	1.08 ± 0.60	2	1.21 ± 0.68	1.18 ± 0.70

* Values are means ± SD (mean ± range for DDATP), calculated from the specific activity data supplied with each radiolabeled analog. 0 DP and 1 μM DP denote the absence and the presence of 1 μM dipyridamole, respectively. Abbreviations: UTP, uridine 5′-triphosphate; AZTTP, azidothymidine 5′-triphosphate; and DDATP, 2′,3′-dideoxyadenosine 5′-triphosphate.

DISCUSSION

We initiated these experiments to examine if there are broad specificity equilibrative nucleoside transport systems in human immune cells that are dipyridamole sensitive (the "*es*" transporter [17]). Our zero-trans uridine influx data suggest that the facilitated nucleoside transport system is most active in lymphocytes and least active in bone marrow mononuclear cells. In human macrophages, there appears to be a dipyridamole-insensitive nucleoside transport component in addition to the traditional dipyridamole-sensitive mode of influx. It has been reported that mouse and rat peritoneal macrophages express solely a Na^+-dependent concentrative nucleoside uptake system that is insensitive to dipyridamole [18]. Similar Na^+-dependent transport systems have been reported in mouse intestinal epithelial cells [17] and other cultured neoplastic cells. Whether the dipyridamole-insensitive uridine uptake component in human macrophages represents such a system awaits further characterization. A facilitated uptake system for cytosine arabinoside, a cytotoxic nucleoside analog, has also been reported in proliferating human thymocytes by Smith and coworkers [19].

Several anti-viral dideoxynucleosides have shown promising activity *in vitro* [20]. These compounds are thought to act by permeating the virally-infected cells and are subsequently converted into their active metabolites by phosphorylation. The active metabolites generally have higher affinities for viral enzymes than the natural substrates. These false precursors, therefore, interfere with nucleic acid synthesis by inhibiting viral reverse transcriptase and cause DNA chain termination after their incorporation into the replicating strands [21, 22].

It was somewhat surprising, however, that no detectable levels of DDI, DDC, and DDT were found inside the three cell types even after 2 hr of equilibration. Such poor cellular penetration may be an important determinant in their anti-HIV efficacy in AIDS patients. DDA, on the other hand, permeated the cells almost as well as uridine within this longer time frame, while AZT was found at somewhat lower levels (Table 2). Although the long-term uptake of uridine was inhibited more than 80% by 1 μM dipyridamole, the uptake of AZT and DDA was not altered by this nucleoside transport inhibitor. Our data are consistent with previous reports that the uptake of AZT [11] and DDA [23] into human cells is not blocked by nucleoside transport inhibitors.

The dideoxynucleoside analogs showed dramatic differences in their ability to inhibit HIV replication *in vitro*. In the ATH8 test system [24], the IC_{50} for DDC was 0.1 μM, whereas for DDT it was 150 μM. It was initially assumed that the 5′-triphosphate of these nucleosides had different affinities for reverse transcriptase. However, Hao *et al.* [25] reported that the 5′-triphosphates of four dideoxynucleosides and AZT are all excellent inhibitors of HIV reverse transcriptase in a cell-free assay with a narrow range of apparent K_i values (0.1 to 0.26 μM). These reports, taken together with our dideoxynucleoside permeation data, suggest that the effectiveness of an anti-HIV dideoxynucleoside is related to its ability to permeate target cells. Future drug development efforts should pay special attention to analogs that are substrates of the membrane nucleoside transporter so that therapeutic levels in target cells can be readily achieved. An example of such a nucleoside analog is cytosine arabinoside [19].

Other research groups have reported that DDC accumulates to low levels in cultured human CEM cells [26] and other tumor cells [27–29]. One study reported that DDC influx into CEM cells is inhibited by nucleoside transport inhibitors such as nitrobenzylthioinosine and dipyridamole [26]. We cannot offer any explanation for the apparent disparity between those results and our present data except to note that the difference may be related to those experiments being performed on transformed lymphoid cells and tumor cell lines, whereas our experiments used normal human immune effector cells. Furthermore, recent data suggest that the degree of *in vitro* toxicity of the dideoxynucleosides in canine bone marrow progenitors is closely correlated to their ability to permeate bone marrow cells [14]. The data reported here imply that if DDC and DDI can inhibit HIV replication in human patients, it may work via extracellular mechanisms. Whether HIV-infected human immune effector cells have the same nucleoside permeation characteristics remains to be examined.

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