Localization of Purine Metabolizing Enzymes in Bovine Brain Microvessel Endothelial Cells: An Enzymatic Blood-Brain Barrier for Dideoxynucleosides?

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Purpose. The specific activities of the purine and pyrimidine metabolizing enzymes, purine nucleoside phosphorylase (PNP), adenosine deaminase (ADA) and cytidine deaminase (CDA) were determined in bovine brain microvessel endothelial cells (BBMECs), whole cerebral tissue and erythrocytes. In addition, the substrate specificities (K_m and V_{max}) of purified calf spleen PNP for inosine and 2′,3′-dideoxyinosine (ddI) and of purified calf intestinal ADA for 2′,3′-dideoxyadenosine (ddA), 6-chloro-2′,3′-dideoxypurine (6-Cl-ddP), and 2′-β-fluoro-2′,3′-dideoxyadenosine (F-ddA) have been explored.

Methods. BBMECs were isolated from bovine cerebral cortex by a two step enzymatic dispersion treatment followed by centrifugation over 50% Percoll density gradients. Activities of alkaline phosphatase, γ-glutamyl transpeptidase, ADA, PNP and CDA were determined in various tissue homogenates (cerebral cortex, BBMECs and erythrocytes). Enzyme kinetic studies were also conducted using commercially available enzymes and several nucleoside analogs of interest.

Results. The activities of ADA and PNP were 42-fold and 247-fold higher in the cerebral microvessels than in the cerebral cortex, respectively, while there was no detectable CDA activity in the microvessel fraction and very little overall activity in the cortex.

Conclusions. ADA and PNP may serve as an enzymatic blood-brain barrier for some of the anti-HIV dideoxynucleosides. Simulations of brain availability for ddI, ddA, 6-Cl-ddP, and F-ddA demonstrated that the quantitative significance of enzyme localization may vary dramatically, however, depending on the membrane permeability of the drug and its bioconversion rate constant within the endothelial cell.

KEY WORDS: 2′,3′-dideoxynucleoside; bovine brain endothelial cell; adenosine deaminase; cytidine deaminase; purine nucleoside phosphorylase; enzymatic blood-brain barrier.

INTRODUCTION

The endothelial cells of the cerebral microvasculature, widely considered to constitute the blood-brain barrier (BBB), function as both a permeability barrier and a metabolic barrier to the passage of chemicals from the blood into the central nervous system (CNS) (1). Recent data indicate that isolated brain capillaries contain significantly higher activities of certain drug metabolizing enzymes than the parenchyma itself, reinforcing the concept of an enzymatic blood-brain barrier (2–4).

The potential localization of purine and pyrimidine metabolizing enzymes in blood-brain barrier capillary endothelia may have important implications in the CNS delivery of a number of purine and pyrimidine 2′,3′- dideoxynucleosides which have been investigated as potential therapeutic agents for the treatment of AIDS or are presently marketed as anti-HIV drugs. Among the commercially available nucleoside analogs, 2′,3′-dideoxyinosine (ddI, didanosine) is a substrate for purine nucleoside phosphorylase (PNP) (5), while 2′,3′-dideoxyadenosine (ddA) and 2′-β-fluoro-dideoxyadenosine (F-ddA), a more acid stable analogue of ddA, are readily deaminated to ddI or 2′-β-fluoro-ddI through the action of adenosine deaminase (ADA) (6). Elevated activities of these enzymes in brain capillary endothelial cells may play a significant role in therapy by either restricting the CNS delivery of 2′,3′-dideoxynucleoside substrates or altering the form which ultimately reaches the brain. Consistent with a plausible role for endothelial cell metabolism, studies in these laboratories have demonstrated a substantially reduced BBB permeability-area product for ddI (in rats) in comparison to that expected from its octanol/water partition coefficient (7). More recently, we have shown that steady-state levels of F-ddA in the rat brain are significantly reduced as a result of ADA catalyzed deamination in brain tissue (8).

The prevalence of AIDS dementia complex, a progressive encephalopathy in which behavioral and motor dysfunction predominate, and its association with human immunodeficiency virus (HIV) infection in the CNS (9), heightens the need to understand both the physical and enzymatic components of the BBB governing the CNS entry of anti-HIV agents. Moreover, elevated levels of certain enzymes in capillary endothelial cells or brain tissue may also be exploited in the design of brain targeted prodrugs or drug-carrier conjugates which undergo brain tissue selective bioconversion. Thus, both ADA (10) and xanthine oxidase (11) activated prodrugs have shown promising potential in improving the CNS delivery of their parent dideoxynucleosides in animal models due, in part, to their facile bioconversion in the brain.

In this report, we have assessed the specific activities of the purine and pyrimidine metabolizing enzymes, PNP, ADA and cytidine deaminase (CDA) in bovine brain microvessel endothelial cells, whole cerebral tissue and erythrocytes. In addition, we have examined the substrate specificities (K_m and V_{max}) of purified calf spleen PNP for inosine and ddI and of purified calf intestinal ADA for ddA, 6-Cl-ddP, and F-ddA. Knowledge of the specific activities of these enzymes localized within the BBB combined with passive permeability coefficient estimates and V_{max} and K_m data allow the assessment of the possible importance of the enzymatic BBB in governing the CNS entry of these compounds.

MATERIALS AND METHODS

Reagents

ddI and ddA were provided by the National Cancer Institute, NIH, and were used as received. L-γ-glutamyl-*p*-nitrophenol, *p*-nitroaniline, glycylglycine, *p*-nitrophenyl phosphate, *p*-nitrophenol, inosine, hypoxanthine, cytidine, uridine, 1-octane-

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ABBREVIATIONS: ADA, adenosine deaminase; BBB, blood-brain barrier; BBMEC, bovine brain microvessel endothelial cells; CDA, cytidine deaminase; HIV, human immunodeficiency virus; PNP, purine nucleoside phosphoryase; 6-Cl-ddP, 6-chloro-2′,3′-dideoxypurine; F-ddA, 2′-β-fluoro-2′3′-dideoxyadenosine; ddA, 2′,3′-dideoxyadenosine; ddI, 2′,3′-dideoxyinosine.

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sulphonic acid, purine nucleoside phosphorylase (purified from calf spleen), and adenosine deaminase (Type VII from calf intestinal mucosa) were purchased from Sigma (St. Louis, MO). [8-^{14}C]Inosine (50 mCi/mmol) and [2-^{14}C]cytidine (55 mCi/mmol) were obtained from Moravek Biochemicals (Brea, CA) and used as received.

Tissue Preparation

Cerebral Cortex

Gray matter devoid of meninges and large surface vessels was collected from bovine cerebral cortices (1–3 animals pooled per experiment) and minced. Portions of this tissue were utilized in the preparation of bovine brain microvessel endothelial cells (below). Cerebral cortex used for enzyme assays was diluted with an equal volume of ice cold sterile water, homogenized at 4°C with a motorized Potter-Elvehjem tissue grinder (0.1–0.15 mm clearance), and stored in small aliquots in sterile cryogenic vials at −70°C for later use.

Bovine Brain Microvessel Endothelial Cells (BBMEC)

Purified microvessel fragments were isolated from bovine cerebral gray matter by a two-step enzymatic (dispase and collagenase-dispase) dispersion treatment followed by centrifugation over pre-established 50% Percoll density gradients as previously detailed by Audus and Borchardt (12). In addition, partially purified microvessel fragments, assumed to contain endothelial cells encased within the basement membrane accompanied by contaminating neuroglial cells (pericytes and astroctyes) (13) were collected after the first enzymatic treatment with dispase II followed by centrifugation over pre-established 50% Percoll density gradients. Prior to use in enzyme assays, isolated capillary microvessels (partially purified and purified) were treated as previously described for whole cerebral tissue. To ensure that the cerebral microvessel fragments used in these experiments consisted primarily of brain capillary endothelial cells, biochemical assays for γ-glutamyl transpeptidase and alkaline phosphatase, two enzymes believed to be specific markers for blood-brain-barrier microvascular endothelium (14), were performed. General morphology was also examined by transmission electron microscopy as described below.

Erythrocytes

Whole blood, collected from five animals at the time of extermination, was placed in a sterile vessel containing a solution of physiologic saline (0.9% NaCl) and heparin (12.5 units/mL). Aliquots of the well mixed solution were centrifuged at 3000g for 15 minutes at 4°C. The supernatant was discarded and the cells were washed in 0.9% NaCl. This procedure was repeated twice, after which the red blood cells were resuspended in an equal volume of sterile water and stored in sterile cryogenic vials at −70°C for later use.

Tissue Characterization

Electron Microscopy

Purified brain capillary isolates were fixed in formalin overnight for electron microscopic examination. Samples were pelleted by centrifugation, fixed in 1.0% osmium tetroxide in 0.1 M Na cacodylate buffer, and dehydrated in a graded series of ethanol solutions. Pellets were embedded in Spur low viscosity embedding medium and polymerized overnight at 60°C. Thin sections were cut with a diamond knife (DuPont, Wilmington, Del.) on a NOVA LKB microtome. Sections were counterstained with uranyl acetate (3 min) and lead citrate (1 min) and examined in a JEOL 100S electron microscope.

Protein Analysis

The estimation of total protein was by a modified Lowry assay (Sigma Diagnostics, St. Louis, MO.) using a deoxycholate-trichloroacetic acid protein precipitation procedure to quantitatively recover soluble and membrane proteins from interfering substances (15).

Enzyme Assays

Alkaline Phosphatase

Alkaline phosphatase activity was measured in tissue samples at 37°C using *p*-nitrophenyl phosphate as substrate (13). Initial rates of *p*-nitrophenol formation were monitored spectrophotometrically at 420 nm (ϵ = 14180 M^{-1} cm^{-1}).

γ-Glutamyl Transpeptidase

Enzymatic activity in tissue samples was measured using the procedure of Orlowski and Meister (16). Initial rates of *p*-nitroaniline formation were monitored spectrophotometrically at 410 nm (ϵ = 8638 M^{-1} cm^{-1}).

Adenosine Deaminase (ADA)

ADA activity was measured in tissue samples using ddA as a reference substrate in pH 7.4 phosphate buffer (8.4 mM) at 37°C. Initial rates of ddI formation were monitored using a modular reversed phase HPLC system (Supelcosil LC-18-S analytical column, 25 cm × 4.6 mm ID, 5 μ (Supelco Inc., Bellefonte, PA)) with a mobile phase containing 5% acetonitrile/95% phosphate buffer (pH 7.4, 8.4 mM) and detection at 254 nm.

Additional kinetic studies were carried out at varying substrate concentrations using either purified calf intestinal ADA or BBMECs in order to determine K_m and V_{max} (or relative V_{max}) values for ddA, 6-Cl-ddP, and F-ddA. The procedures were similar to those described above (pH 7.4, 37°C) but with substrate concentrations varying from well below K_m ($\approx 0.1K_m$) to 2–10 times K_m. Initial rates of ddI formation were monitored as previously described. Initial velocity data were fit to the conventional Michaelis-Menten relationship via unweighted nonlinear regression analysis (Scientist, Micromath Inc., SLC, Ut) to obtain estimates of the kinetic parameters K_m and V_{max}.

Purine Nucleoside Phosphorylase (PNP)

Enzymatic activity was measured using inosine as a reference substrate in pH 7.4 phosphate buffer (8.4 mM) at 37°C. Initial rates of hypoxanthine formation were monitored using the HPLC system described above and a mobile phase containing 1% acetonitrile/99% phosphate buffer (pH 7.4, 8.4 mM).

In whole cerebral homogenate, initial rates of hypoxanthine formation were monitored using a combination of HPLC and radiolabeled inosine (0.4 μCi/ml) as impurities precluded the use of HPLC alone. Samples containing hypoxanthine (8-^{14}C) were analyzed with a Beckman LS 1801 scintillation counter (Beckman Instruments Inc., Fullerton, CA.).

Additional kinetic studies were carried out using purified calf spleen PNP in order to determine K_m and V_{max} values for inosine and ddI. The procedures were similar to those described above but with substrate concentrations varying from well below K_m ($\approx 0.1K_m$) to 2–3 times K_m. Initial velocity data were handled in the same manner as previously described.

Cytidine Deaminase (CDA)

Enzymatic activity was measured using cytidine as a reference substrate in pH 7.4 phosphate buffer (8.4 mM) at 37°C. Initial rates of uridine formation were monitored using the HPLC system described previously and a mobile phase containing 100 mM ammonium acetate with 1% (v/v) methanol and 1 mM 1-octanesulphonic acid adjusted to pH 5.0 (17). In whole cerebral homogenate, initial rates of uridine formation were monitored using a combination of HPLC and radiolabeled cytidine (2.5 μCi/ml) as impurities precluded the use of HPLC alone. Samples containing uridine (2-^{14}C) were analyzed with a Beckman LS 1801 scintillation counter (Beckman Instruments Inc., Fullerton, CA).

RESULTS AND DISCUSSION

Purified bovine brain capillaries were isolated and enzymatically treated to remove the collagen-containing endothelial basement membrane and contaminating pericytes and astrocytes embedded within and around it. Subsequent Percoll gradient centrifugation separated the microvessels from other cells and debris. Phase contrast microscopy of the isolates revealed continuous tufts of intact capillary segments relatively free from contaminating cells. Electron microscopic examination of the capillaries revealed endothelial cells with intact lumenal and ablumenal plasma membranes, an intact nucleus and a small number of pinocytotic vesicles. Cytoplasmic organelles, such as mitochondria, were also present and generally well preserved. Enzyme activity assays for the blood-brain barrier specific marker enzymes, γ-glutamyl transpeptidase and alkaline phosphatase, indicated that these membrane-associated enzymes of the capillary endothelial cells were enriched 11-fold and 33-fold respectively when compared to cerebral cortex homogenate (Table I).

Localization of Purine Metabolizing Enzymes

The activities of PNP, ADA and CDA in brain capillaries (partially purified and purified), cerebral cortex and erythrocytes, which were determined from the initial rates of hypoxanthine, ddI and uridine formation, respectively, are listed in Table I. While the specific activities of ADA and PNP in the partially purified BBMEC fraction (containing both endothelial and neuroglial cells) increased 14-fold and 63-fold relative to the cerebral cortex, they were 3 to 4-fold lower than the respective specific activities in the purified BBMEC fraction containing mostly cells of endothelial origin, suggesting that an enzymatic barrier to dideoxynucleoside transport may indeed exist at the level of the endothelial cells. The enrichment factors for each enzyme, which are conventionally defined as the ratio of specific activity in brain microvessel endothelial cell to that in cerebral cortex (BBMEC/CC), are also listed in Table I. Based on this convention, PNP and ADA are enriched 247-fold and

Table I. Enzyme Activity in Bovine Brain Microvessel Endothelial Cells (BBMECs), Cerebral Cortex, and Erythrocytes

Enzyme	Specific Activity[a]				Enrichment Factor (BBMEC/CC)
	BBMEC (purified)	BBMEC (partially purified)	Cerebral Cortex (CC)	Erythrocytes	
γ-Glutamyl transpeptidase[b]	35 ± 4 (2)	ND[e]	3.3 ± 0.3 (2)	ND[e]	11:1
Alkaline phosphatase[b]	460 ± 99 (3)	ND[e]	14 ± 2 (2)	ND[e]	33:1
Adenosine deaminase[b]	216 ± 56 (3)	75 ± 2 (2)	5.2 ± 0.3 (2)	0.015 ± 0.002 (4)	42:1
Cytidine deaminase[c]	NA[d]	NA[d]	0.5 ± 0.3 (2)	7.8 ± 0.2 (2)	0:1
Purine nucleoside phosphorylase[b]	74 ± 13 (2)	19 ± 0.5 (2)	0.3 ± 0.1 (2)	12 ± 1 (2)	247:1

[a] Reported values for enzyme activity in purified brain capillaries and cerebral cortex are the averages ± SD of several mean values evaluated from replicate measurements (2–4) of enzyme activity in pooled tissue samples representing 1–3 animals. Listed in parentheses are the number of groups contributing to the reported average. Values for enzyme activity in partially purified microvessels and erythrocytes are the means ± SD from replicate measurements within a single group where the values in parentheses are the number of replicate measurements contributing to the reported average.

[b] Activity is expressed in nmole product formed per minute per milligram protein at 37°C. Substrates: 2.7 mM L-γ-glutamyl-p-nitroanilide (γ-glutamyl transpeptidase), 1.8 mM *p*-nitrophenyl phosphate (alkaline phosphatase), 0.45 mM ddA (adenosine deaminase), 0.27 mM inosine (purine nucleoside phosphorylase).

[c] Activity is expressed in pmole product formed per minute per milligram protein at 37°C using 0.037 mM cytidine as substrate.

[d] No activity.

[e] Not determined.

42-fold, respectively, while no CDA activity could be detected in brain capillary endothelial cells.

PNP catalyzes the reversible phosphorolysis of the purine nucleoside inosine and the 2′,3′-dideoxynucleoside ddI in the presence of inorganic phosphate to yield the nucleobase hypoxanthine and ribose 1-phosphate or 2′,3′-dideoxyribose 1-phosphate, respectively (18). The kinetic parameters obtained from best fits of the initial velocity versus concentration data to the Michaelis-Menten equation, displayed in Table II, provide a quantitative evaluation of substrate specificity. Consistent with the conclusions drawn by Stoeckler *et al.* regarding the significance of the 2′-OH of the carbohydrate moiety to the binding of the nucleoside to the active site of PNP and its subsequent reactivity (18), ddI exhibited a 10-fold increase in K_m and a marked decrease in V_{max} relative to inosine.

ADA is a key enzyme in purine metabolism. In addition to catalyzing the irreversible hydrolytic deamination of adenosine to produce inosine and ammonia, this enzyme also catalyzes the hydrolysis of several analogs of adenosine altered in either the purine ring or sugar moiety and 6-substituted purine derivatives (19–21). In this study, ddA was used as a reference substrate as its K_m and V_{max} are similar to those of adenosine (19, 20). Michaelis-Menten behavior over an extended range of substrate concentrations was observed in both purified calf intestinal ADA containing systems and BBMECs, with similar Km values. Table II gives a summary of the kinetic parameters obtained from best fits of the initial velocity versus concentration data to the Michaelis-Menten equation using purified ADA. Relative to ddA, both F-ddA and 6-Cl-ddP exhibit increased K_m and decreased V_{max} values such that the V_{max}/K_m ratio is substantially decreased for these substrates.

Does the BBB Function as an Enzymatic Barrier for Dideoxynucleosides?

There appear to be a host of metabolic enzymes localized within the capillary endothelial cells which may constitute an enzymatic or "metabolic" blood-brain barrier (2–4). In this report, we have quantified the activity of two key purine metabolizing enzymes and have found their activities to be significantly higher in capillary endothelial cells relative to whole cerebral gray matter. Our results suggest that a potential metabolic barrier to dideoxynucleoside brain entry resides at the level of the endothelial cells of the cerebral microvasculature. Additionally, we have discovered that the pyrimidine metabolizing enzyme, CDA, does not appear to be highly localized within the microvasculature of the cerebral gray matter. CDA is present in brain tissue but its distribution is largely unknown (22).

To assess the significance of BBB metabolism in the CNS delivery of 2′,3′-dideoxynucleosides which are substrates of either ADA or PNP, the kinetic model illustrated in Figure 1 provides useful insight. In this simplified model, the initial rate of uptake of drug from blood into the brain parenchyma is assumed to be governed by its permeability-area product (P_mA) and metabolic clearance within the endothelial cell ($Cl_{e.c.} = k_{e.c.}*V_{e.c.}$). In the absence of a contribution from carrier-mediated processes, membrane permeability (P_m) can be estimated from the octanol/water partition coefficient of the permeant (23) while the brain capillary surface area, A, is a constant independent of the permeant. Allowing the drug concentration within the endothelial cell to be at steady-state and assuming the backflux of drug into the intracellular space to be negligible (initial uptake region), the fraction of drug leaving the plasma which enters the brain parenchyma intact can be expressed as (see Appendix for derivation):

$$\text{availability} = \frac{P_m}{P_m + k_{e.c.}\frac{V_{e.e.}}{A}} \quad (1)$$

where $k_{e.c.}$ ($= V_{max}/K_m$) is the first-order bioconversion rate constant in the endothelial cell cytoplasm and $V_{e.c.}$ is the intracellular volume of endothelial cells within the brain. Pardridge (24) has estimated that in the human brain the capillary endothelial cell intracellular volume is approximately 1 cc and the total capillary surface area is approximately 100 square feet (10^5 cm^2), yielding a $V_{e.c}/A$ ratio of 10^{-5} cm. If this ratio is species independent, the same value would apply in Eq. [1].

Eqn. [1] implies that drug availability to the brain is determined by the relative magnitudes of P_m and $k_{e.c.}$. Examining the enzyme activities in purified brain endothelial cells (Tables

Table II. Enzyme Kinetic Parameters at pH 7.4 and 37°C for Nucleoside and Dideoxynucleoside Substrates of Purine Nucleoside Phosphorylase and Adenosine Deaminase

Compound	Bioconversion System	Kinetic Parameters: K_m^a (μM)	Kinetic Parameters: Relative V_{max}
Inosine	PNP[b]	145 ± 3	100%[d]
ddI	PNP[b]	1470 ± 160	6.2%
ddA	ADA[c]	107 ± 17	100%[e]
6-Cl-ddP	ADA[c]	12200 ± 900	71%
2′-F-ddA	ADA[c]	280 ± 60	16%

[a] K_m values (estimate ± SD) were determined from computer fits of the initial velocity versus concentration data to the Michaelis-Menten equation.
[b] Purified purine nucleoside phosphorylase from calf-spleen.
[c] Purified calf intestinal adenosine deaminase.
[d] $V_{max} = 47 \pm 3$ μmol min^{-1} $unit^{-1}$.
[e] $V_{max} = 0.96 \pm 0.26$ μmol min^{-1} $unit^{-1}$.

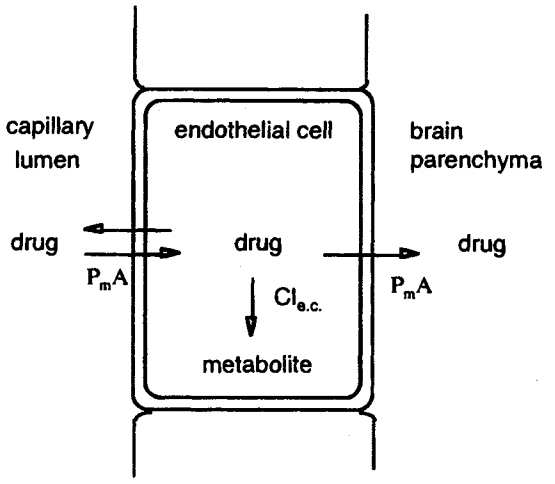

Fig. 1. Diagram illustrating the enzymatic and transport components of the blood-brain barrier.

I & III) and assuming that endothelial cells contain approximately 100 mg protein/ml of intracellular volume, one can obtain endothelial cell V_{max} estimates which, when divided by the K_m values listed in Table II, provide estimates of $k_{e.c.}$ in endothelial cells. These estimates are listed for ddI, ddA, F-ddA and 6-Cl-ddP in Table III. Also shown in Table III are octanol/water partition coefficients for these compounds from which permeability coefficients were calculated according to the relationship developed by Rapaport *et al.* (23). Finally, estimates of brain availability according to Eqn. [1] are listed.

The availability estimates in Table III show that even though ADA and PNP are localized within the BBB, the enzymatic component of the barrier for substrates of these enzymes may or may not be significant, depending on the specific substrate in question. Thus, though ddI would be expected to exhibit a relatively low permeability coefficient based on its octanol/water partition coefficient, it is also a relatively poor substrate for PNP. The availability estimate for ddI of >99% obtained in Table III suggests that factors other than an enzymatic blood-brain barrier need to be considered to account for its reduced CNS uptake *in vivo* (7, 25, 26). On the other hand, though ddA is expected to exhibit a significantly higher BBB permeability coefficient than ddI due to its enhanced lipophilicity, the rate constant for ADA catalyzed bioconversion of ddA in endothelial cells is substantial. This "first-pass" BBB metabolism may lead to a significant reduction in the fraction of ddA reaching the brain parenchyma intact.

In conclusion, this study has shown that two important enzymes in the purine salvage pathway, ADA and PNP are highly localized in the capillary endothelial cells within bovine brain tissue. These enzymes may serve as an enzymatic blood-brain barrier for some of the anti-HIV dideoxynucleosides. However, simulations of brain availability for ddI, ddA, 6-Cl-ddP, and F-ddA have demonstrated that the quantitative significance of enzyme localization is highly variable, as it is determined by the competition between membrane permeability, governed by drug lipophilicity, and the endothelial cell bioconversion rate constant, which depends on many factors, including the enzyme activity in the endothelial cell and the substrate's K_m and V_{max}. In extrapolating these results to humans, one must bear in mind the significant species-to-species variability in tissue activity of these enzymes (27). Notably, brain tissue levels of ADA appear to be relatively high in humans compared to some other species (28).

Table III. Physicochemical Properties of Various Nucleoside Analogs and Estimates of Brain Availability (Eq. [1])

Compound	O/W PC	P_m (cm min^{-1})[d]	Relative V_{max}/K_m	$k_{e.c.}$ (min^{-1})	Percent Available
Inosine	ND[a]	ND[a]	1.0	78	ND[a]
ddI	0.058[b]	5.2e-4	0.006	.5	>99%
ddA	0.52[b]	3.4e-3	1.0	250	58%
2'-F-ddA	0.66[b]	4.2e-3	0.06	15	97%
6-Cl-ddP	1.7[c]	9.6e-3	0.005	1.2	>99%

[a] Not determined.
[b] Reference (29).
[c] Reference (30).
[d] From P values (log P = −4.30 + 0.866 log PC) of Rapoport *et al.* (23). P_m = 2P since transport across 2 membranes is required (Fig. 1) for entry into brain parenchyma.

APPENDIX

Derivation of Eq. (1)

The CNS availability of a substrate is defined as the ratio of its rate of appearance in the brain parenchyma (dX_{br}/dt) to its rate of loss from plasma attributable to passage across the blood-brain barrier, $(-dX_p/dt)_{BBB}$. Thus,

$$\text{availability} = \frac{(dX_{br}/dt)}{(-dX_p/dt)_{BBB}} \quad (2)$$

where the rates of entry into brain and loss from plasma can be expressed (see Fig. 1) in terms of the membrane permeability coefficient (P_m), blood-brain barrier surface area (A), and endothelial cell and plasma substrate concentrations ($C_{e.c.}$ and C_p, respectively):

$$(dX_{br}/dt) = P_m A C_{e.c.} \quad (3)$$

$$(-dX_p/dt)_{BBB} = P_m A(C_p - C_{e.c.}) \quad (4)$$

Assuming steady-state within the endothelial cell and sink conditions within the brain parenchyma (i.e., initial uptake region), $C_{e.c.}$ can be expressed as:

$$C_{e.c.} = \frac{P_m A C_p}{2P_m A + k_{e.c.} V_{e.c.}} \quad (5)$$

where $V_{e.c.}$ is the total endothelial cell volume and $k_{e.c.}$ is the first-order metabolic rate constant within the endothelial cell ($= V_{max}/K_m$). Inserting Eq. 5 into Eqs. 3 & 4 and then taking their ratio leads to Eq. 1.

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