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INHIBITORY EFFECTS OF PROPENTOFYLLINE ON [^{3}H]ADENOSINE INFLUX

A STUDY OF THREE NUCLEOSIDE TRANSPORT SYSTEMS

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Abstract—The neuroprotective effects of adenosine are well-recognized. Recently, propentofylline, a xanthine derivative, has been shown to increase extracellular concentrations of adenosine in ischemic brain and to limit the extent of neuronal damage in experimental models of cerebral ischemia. Since the concentration of adenosine in brain is controlled, in part, by nucleoside transporter proteins, the action of propentofylline was proposed to be due to inhibition of mediated transfer of adenosine across cell membranes. To determine the likelihood of this mechanism, we examined the inhibitory effects of propentofylline on [^{3}H]adenosine transport by the three best-characterized nucleoside transport processes, *es*, *ei*, and *cif* in cultured cell lines under conditions where only a single transporter type was operative. Propentofylline inhibited [^{3}H]adenosine uptake by each of the three transport processes in a concentration-dependent manner. The greatest inhibitory potency was for *es* transporters (L12'0/B23.1 cells), with an IC_{50} value of 9 μM, followed by *ei* transporters, with IC_{50} values of 170 μM (L1210/C2 cells) and 166 μM (Walker 256 cells). Propentofylline was a weak inhibitor of *cif* transporter, with an IC_{50} value of 6 mM. These results demonstrate that propentofylline is an inhibitor of adenosine transport processes and suggest that its neuroprotective effects may be due to an increase in extracellular concentrations of adenosine by virtue of inhibition of *es* transporter function.

The extracellular levels of adenosine rapidly increase in brain following hypoxia or ischemia [1–4]. This released adenosine, by interacting with adenosine A_1 receptors, decreases neurotransmitter release and neuronal firing [5]. Much evidence has accumulated to support the neuroprotective effects of adenosine A_1 receptor agonists in cerebral ischemia [6–9], although systemic administration of these compounds produces severe cardiovascular side-effects [10, 11].

Propentofylline is a novel xanthine compound that has been shown to limit the extent of neuronal damage in experimental models of cerebral ischemia [3, 4, 12]. Propentofylline reduces accumulation of [^{3}H]adenosine into human erythrocytes [13] and increases extracellular concentrations of adenosine in ischemic brain [4]. It has been suggested that inhibition of the transport of adenosine into cells, which could raise extracellular adenosine levels and thereby potentiate the receptor-mediated effects of endogenously produced adenosine, may account for the cerebroprotection induced by propentofylline [3, 4, 13–15]. Both influx and efflux of adenosine are mediated by *es* transporters; however, during conditions that increase intracellular concentrations of adenosine, such as ischemia, inhibitors of transport may have a greater effect on influx [see Ref. 16].

Nucleoside transport systems are classified as equilibrative (Na^+-independent) or concentrative (Na^+-dependent) processes. These processes are further subdivided according to sensitivity to the inhibitor nitrobenzylthioinosine (NBMPR)‖ and substrate selectivity. Thus, the equilibrative processes include *es*, which is *e*quilibrative and *s*ensitive to inhibition by NBMPR, and *ei*, which is *e*quilibrative and *i*nsenstive to NBMPR [17]. Na^+-dependent nucleoside transport systems include the purine-selective *cif* (*c*oncentrative, *i*nsensitive to NBMPR, *f*ormycin B selective; also called N1), the pyrimidine-selective *cit* (*c*oncentrative, *i*nsensitive to NBMPR, *t*hymidine selective; also called N2) and the nonselective *cib* (*c*oncentrative, *i*nsensitive to NBMPR, *b*road substrate selectivity) [17–20]. Although the Na^+-dependent systems are classified according to substrate specificity, all of those identified to date accept adenosine as a permeant.

Tissue preparations from brain express several coexisting nucleoside transport processes that are difficult to resolve into individual components [see Ref. 21]. Considering the neuroprotection afforded by propentofylline, knowledge of the nucleoside transport systems inhibited by propentofylline may indicate which systems are important in regulating the concentration of endogenous adenosine in areas of the brain vulnerable to ischemic damage. Therefore, in the present study, we examined the inhibition by propentofylline of [^{3}H]adenosine transport by the three best-characterized nucleoside

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‖ Abbreviations: NBMPR, nitrobenzylthioinosine; and NMG^+, *N*-methyl-D-glucammonium.

transport systems: *es*, *ei* and *cif*, using cultured cell lines with well-characterized nucleoside transport systems and conditions where only a single transporter was operative. Clonal mouse leukemia L1210/C2 cells, which possess all three systems (*es*, *ei*, and *cif*) [22], were used to assay *ei* transporters by blocking *es* (with NBMPR) and *cif* (by replacing Na^+). Mutation strategies have led to the isolation of L1210/B23.1 cells that express only *es* [23] and L1210/MA27.1 cells that possess only *cif* [24], and these lines were used to assay *es* and *cif*, respectively. Walker 256 rat carcinosarcoma cells, which possess both *ei* and *cif* [25], were also used to assay *ei* transport.

MATERIALS AND METHODS

Materials. [^{3}H]Adenosine and [^{3}H]NBMPR were purchased from Moravek Biochemicals (Brea, CA) and, after storage, were purified by high performance liquid chromatography to greater than 98% purity. ^3H_2O (5 mCi/mL) was from Amersham Canada Ltd. (Oakville, Ontario) and [^{14}C]polyethylene glycol (20 mCi/g) was from DuPont Canada (Mississuaga, Ontario). NBMPR, dipyridamole, adenosine, *N*-methyl-D-glucamine and Triton X-100 were purchased from the Sigma Chemical Co. (St. Louis, MO). RPMI 1640 and heat-inactivated horse serum were obtained from Gibco BRL (Burlington, Ontario). Dilazep was provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Propentofylline was a gift from Dr. K. A. Rudolphi, Hoechst AG (Wiesbaden, F.R.G.). L1210/MA27.1 cells were provided by Dr. J. A. Belt.

Cell culture. Cells (L1210/C2, L1210/B23.1, L1210/MA27.1 and Walker 256) were maintained as exponentially proliferating cultures at cell densities of 0.5×10^5 to 5×10^5 cells/mL in RPMI 1640 culture medium supplemented with 10% heat-inactivated horse serum. The transport properties of these cell lines have been described [22–25].

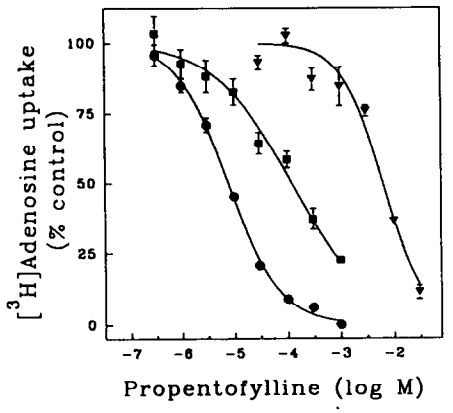
For transport assays, cells were harvested by centrifugation (100 *g*, for 10 min) and resuspended (10^6 cells/mL) in Na^+ buffer (in mM: Tris, 20; K_2HPO_4, 3; NaCl, 120; $MgCl_2$, 1.0; $CaCl_2$, 1.2; to pH 7.4 with HCl) containing 10 mM glucose and incubated for 5–10 min at 22° prior to initiation of uptake intervals. For experiments performed in low Na^+, cells were treated similarly, except that they were washed three times with *N*-methyl-D-glucammonium (NMG^+) buffer (in mM: Tris, 20; K_2HPO_4, 3; *N*-methyl-D-glucamine, 120; $MgCl_2$, 1.0; $CaCl_2$, 1.2; to pH 7.4 with HCl) containing 10 mM glucose. Osmolarity of the buffers was adjusted, as necessary, to 300 ± 10 mOsmol with NaCl or *N*-methyl-*p*-glucamine.

[^{3}H]*Adenosine influx measurements.* [^{3}H]Adenosine uptake was determined by an oil-stop centrifugation method [26]. Briefly, a reaction mixture (100 μL) containing 1 μM [^{3}H]adenosine 2.5 μCi/mL) in either Na^+ or NMG^+ buffer was layered over oil (85 parts silicon oil : 15 parts paraffin oil; 200 μL) in a microcentrifuge tube. Uptake was initiated by rapid addition of the cell suspension (100 μL) to the reaction mixture and was terminated by rapid addition of an ice-cold stop solution (200 μL), containing 200 μM dilazep (L1210/B23.1 cells) or 40 μM dipyridamole and 2 mM adenosine (L1210/C2 and Walker 256 cells) followed immediately by centrifugation of the cells through oil (16,000 *g* 30 sec). Na^+-dependent transport in L1210/C2 cells is not inhibited by dilazep or dipyridamole [27]; therefore, uptake intervals with L1210/MA27.1 cells were terminated by centrifugation alone, as described by Crawford *et al.* [24]. Non-mediated [^{3}H]adenosine accumulation was estimated with cells preincubated for 15 min with 10 μM NBMPR (L1210/B23.1 cells), 40 μM dipyridamole and 2 mM adenosine (Walker 256 and L1210/C2 cells) or cells prepared in NMG^+ buffer (L1210/MA27.1 cells). The supernatant fractions were aspirated, the tubes were washed twice with water, the oil was removed, and the pellets were dissolved in 5% Triton X-100 for determination of radioactivity by liquid scintillation counting. The extracellular space of cell pellets was determined with cell suspensions exposed to a reaction mixture containing [^{14}C]polyethylene glycol (2 μCi/mL), and the total water space was determined with a reaction mixture containing ^3H_2O (1 μCi/mL). The intracellular water volume was taken as the difference between the ^3H_2O volume and the [^{14}C]polyethylene glycol volume and was (mean ± SEM) 0.34 ± 0.06 pL/cell (N = 6) for L1210/B23.1 cells, 0.41 ± 0.08 pL/cell (N = 6) for L1210/MA27.1 cells, 0.31 ± 0.04 pL/cell (N = 17) for L1210/C2 cells, and 0.69 ± 0.05 pL/cell (N = 6) for Walker 256 cells.

Inhibition of [^{3}H]*adenosine influx.* Experiments were performed as described above except that reaction mixtures contained graded concentrations of propentofylline or NBMPR. For some experiments, cells were preincubated (15 min) with inhibitor; the concentrations of inhibitor used in the preincubations were equal to those in the final assay mixtures. The time intervals used for assessing inhibition of [^{3}H]adenosine influx by propentofylline were chosen based on the results of time course experiments and were 4 sec in L1210/B23.1 cells, 8 sec in Walker 256 cells and L1210/MA27.1 cells, and 20 sec in L1210/C2 cells. The time interval used for assessing inhibition of [^{3}H]adenosine influx by NBMPR in L1210/C2 cells was 8 sec.

Inhibition of [^{3}H]*NBMPR binding by propentofylline.* [^{3}H]NBMPR binds with high affinity and high site density to L1210/B23.1 cells (K_d, 0.1 nM; B_{max}, 100,000 sites/cell) [23]. The affinity of propentofylline for [^{3}H]NBMPR binding sites was determined in equilibrium binding experiments. L1210/B23.1 cells were incubated with 0.25 nM [^{3}H]NBMPR alone or with graded concentrations of propentofylline (300 nM to 10 mM) for 30 min (22°). Site-specific binding of [^{3}H]NBMPR was the difference between binding in the absence and presence of dipyridamole (20 μM) [14, 28].

Data analysis. All measurements were in triplicate, and each experiment was performed at least three times. In experiments where time courses of uptake were obtained, linear regression was used to calculate the initial rates of [^{3}H]adenosine uptake. The IC_{50} values were determined from inhibition experiments using nonlinear regression analysis. Inhibition constants (K_i values) for inhibition of [^{3}H]NBMPR



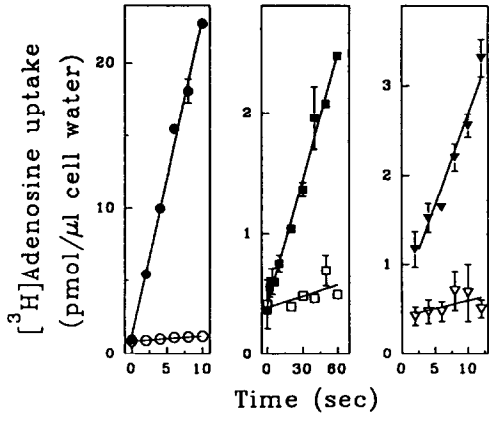


Fig. 1. Top panel: Concentration-dependent inhibition by propentofylline of [3H]adenosine influx into L1210 cells. L1210/B23.1 (●), L1210/C2 (■) or L1210/MA27.1 (▼) cells were used to examine *es*, *ei*, and *cif* transport processes, respectively. *Es*-mediated (control) uptake of [3H]adenosine was the difference between that which occurred in the absence and that which occurred in the presence of 10 μM NBMPR (100% = 7.95 pmol/μL cell water). *Ei*-mediated (control) uptake of [3H]adenosine was the difference between that which occurred in the absence and that which occurred in the presence of 20 μM dipyridamole and 1 mM adenosine (100% = 0.85 pmol/μL cell water). *Cif*-mediated (control) uptake of [3H]adenosine was the difference between that which occurred in Na^+ buffer and that which occurred in NMG^+ buffer (100% = 5.13 pmol/μL cell water). Points are means of triplicates; SEM bars are shown unless obscured by the symbols. Data are from representative experiments. Bottom panel: Time courses of [3H]adenosine uptake by *es*, *ei* and *cif* transport processes in L1210 cells. Left panel: time courses of *es*-mediated [3H]adenosine uptake in L1210/B23.1 cells in the absence (●) or presence (○) of 10 μM NBMPR. Center panel: time courses of *ei*-mediated [3H]adenosine uptake in L1210/C2 cells in the absence (■) or presence (□) of 20 μM dipyridamole and 1 mM unlabelled adenosine. Right panel: time courses of *cif*-mediated [3H]adenosine uptake in L1210/MA27.1 cells in Na^+ buffer (▼) or NMG^+ buffer (▽). Points are means of triplicates; SEM bars are shown unless obscured by the symbols. Data are from representative experiments.

Table 1. Inhibition by propentofylline of the transport of [3H]adenosine into cultured cells

Transport process	Cell line	IC_{50}* (μM)
es	L1210/B23.1	9 (7–12)
ei	L1210/C2	170 (32–912)
cif	L1210/MA27.1	6000 (4000–9000)
ei	Walker 256	166 (124–222)

* Geometric mean (95% confidence interval).

binding to L1210/B23.1 cells by propentofylline were obtained by nonlinear regression analysis and application of the Cheng and Prussoff equation [29], using [3H]NBMPR concentrations corrected for depletion and a K_d value of 0.1 nM [23]. Nonlinear regression analysis was performed with the commercial software package GRAPHPAD INPLOT version 3.1.

RESULTS

The effect of propentofylline on [3H]adenosine transport by the *es* transporter was investigated in a clonal cell line (L1210/B23.1) that expresses only *es* activity (Fig. 1, top panel; Table 1). Uptake of 1 μM [3H]adenosine was linear for up to 10 sec, with an initial rate (mean ± SEM) of 2.0 ± 0.07 pmol/μL cell water/sec. In the presence of 10 μM NBMPR the rate of uptake was not significantly different from zero (Fig. 1, bottom panel). Propentofylline inhibited *es*-mediated uptake of [3H]adenosine with an IC_{50} value (geometric mean with 95% confidence interval) of 9 (7–12) μM (Fig. 1, top panel; Table 1). Preincubation of cells with propentofylline for 15 min (data not shown) had no effect on its ability to block *es* transport of adenosine; the IC_{50} value was unchanged at 9 (6–12) μM.

NBMPR is a nucleoside transport inhibitor that binds with high affinity to the *es* transporter. In many cell types, the ability of an agent to inhibit [3H]NBMPR binding corresponds closely to its ability to inhibit *es*-mediated transport [30–33]. Propentofylline inhibited the binding of [3H]NBMPR to L1210/B23.1 cells with a K_i value of 37 (34–41) μM (Fig. 2), suggesting that the inhibition of [3H]adenosine transport was the result of a specific interaction with the [3H]NBMPR binding site.

Prior to determining the potency of propentofylline against the *ei* transporter of clonal L1210/C2 cells, which coexpresses *es*, *ei*, and *cif*, the concentration dependence of NBMPR inhibition of *es*-mediated transport was examined. In the absence of transport inhibitors, L1210/C2 cells in NMG^+ buffer exhibited an initial rate of uptake of [3H]adenosine, which was linear for up to 10 sec, of 1.0 ± 0.1 pmol/μL cell water/sec (data not shown). In the presence of excess dipyridamole, which inhibits both *es* and *ei* transport activity, and unlabelled adenosine, the rate of [3H]adenosine uptake was not significantly different from zero. NBMPR inhibition exhibited an IC_{50} value of 0.6 (0.07 to 5.8) nM in cells preincubated

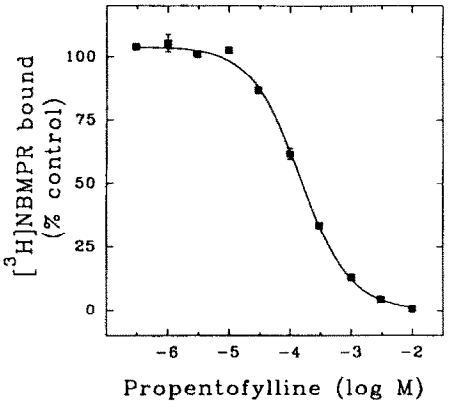


Fig. 2. Concentration-dependent inhibition of [^{3}H]NBMPR binding by propentofylline. L1210/B23.1 cells were incubated with 0.25 nM [^{3}H]NBMPR alone or together with graded concentrations of propentofylline. Site-specific (control) binding of [^{3}H]NBMPR was taken as the difference between binding in the absence and presence of 20 μM dipyridamole (100% = 33,000 molecules/cell). Points are means of triplicates; SEM bars are shown unless obscured by the symbols. Data are from a representative experiment.

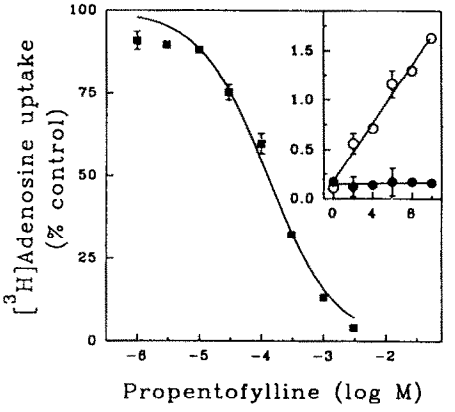


Fig. 3. Inhibition of *ei*-mediated [^{3}H]adenosine influx into Walker 256 cells by propentofylline. Cells were washed and resuspended in NMG^+ buffer after which they were exposed to 1 μM [^{3}H]adenosine alone or with graded concentrations of propentofylline for 8 sec as described in Materials and Methods. *Ei*-mediated (control) uptake of [^{3}H]adenosine was the difference between that which occurred in the absence and that which occurred in the presence of 20 μM dipyridamole and 1 mM adenosine (100% = 2.04 pmol/μL cell water). The *inset* shows typical time courses in the absence (○) and in the presence (●) of 20 μM dipyridamole and 1 mM unlabeled adenosine [*abscissa*, time (sec); *ordinate*, [^{3}H]adenosine uptake (pmol/μL cell water)]. Points are means of triplicate values; SEM bars are shown unless obscured by the symbols. Data are from representative experiments.

with graded concentrations of NBMPR (data not shown).

The equilibrative, NBMPR-insensitive (*ei*) component of [^{3}H]adenosine transport was assayed in L1210/C2 cells preincubated, then incubated, in the presence of 1 μM NBMPR, a concentration that does not significantly inhibit *ei*-mediated transport [22, 32, 34]. This component of transport was very small, amounting to less than 10% of *es*-mediated transport. Therefore, the effects of propentofylline on the *ei*-mediated component of [^{3}H]adenosine uptake were examined over extended intervals (Fig. 1, top panel; Table 1). Uptake was linear over 60 sec, with a rate of 0.041 ± 0.009 pmol/μL cell water/sec and, in the presence of dipyridamole and excess unlabelled adenosine, was not significantly different from zero (Fig. 1, bottom panel). Propentofylline inhibited NBMPR-insensitive [^{3}H]-adenosine uptake with an IC_{50} value of 170 (32–912) μM (Fig. 1, top panel; Table 1).

Walker 256 rat carcinosarcoma cells were also used to determine the affinity of propentofylline for the *ei* subtype of nucleoside transport (Fig. 3, Table 1). Since Walker 256 cells also exhibit *cif* transport activity [25], uptake assays were conducted in Na^+-free buffer. Uptake was linear for up to 10 sec with a rate of 0.1 ± 0.004 pmol/μL cell water/sec and was inhibited completely by dipyridamole and excess unlabelled adenosine (Fig. 3, inset). A concentration-dependent inhibition of [^{3}H]adenosine influx by propentofylline was observed (Fig. 3), with an IC_{50} value of 166 (124–222) μM. Preincubation of Walker 256 cells with graded concentrations of propentofylline had no effect on the IC_{50} value obtained (N = 1; data not shown).

The concentration-dependent inhibition of the *cif* transporter by propentofylline was investigated using L1210/MA27.1 cells which express only the *cif* nucleoside transport system (Fig. 1, top panel, Table 1). The rate of [^{3}H]adenosine uptake in Na^+ buffer was 0.2 ± 0.02 pmol/μL cell water/sec and was constant for up to 12 sec. Replacing Na^+ in the buffer with NMG^+ inhibited [^{3}H]adenosine influx completely (Fig. 1, bottom panel). Propentofylline produced a concentration-dependent inhibition of [^{3}H]adenosine uptake in Na^+ buffer, with an IC_{50} value of 6 (4–9) mM (Fig. 1, top panel; Table 1).

DISCUSSION

Propentofylline, administered either before or up to 1 hr after experimental global ischemia, is cerebroprotective and produces similar neuroprotection to adenosine receptor agonists [3, 4, 6–8, 10, 12, 35]. Because propentofylline inhibits long-term accumulation of [^{3}H]adenosine by isolated human red blood cells, Fredholm and Lindström [13] proposed that it could block removal of adenosine from the vicinity of its receptors and thus enhance the activity of endogenously produced adenosine. The effects of propentofylline in total forebrain ischemia are important because they suggest that inhibition of adenosine uptake may provide part of a therapeutic strategy for the treatment of cerebral ischemia. The neuroprotective effects of established nucleoside transport inhibitors have not been evaluated in experimental models of

cerebral ischemia due to poor penetration of the blood–brain barrier [36, 37].

The present study was performed to confirm that propentofylline inhibits adenosine transport and to determine which of several nucleoside transport systems is most affected. Although nucleoside transport processes of brain have not been fully characterized, the equilibrative systems appear to predominate [32, 33, 38]. Na^+-dependent processes are a small component (<10%) of total nucleoside transport in dissociated brain cells from rat and guinea pig and are not expressed in preparations of dissociated brain cells of mice [39, 40]. Thus, our studies concentrated on *es* and *ei* transport systems.

We found that propentofylline inhibited [^{3}H]-adenosine influx by the three nucleoside transport systems tested. The greatest inhibitory potency was for the *es* transporter, with an IC_{50} value of 9 μM, followed by *ei* transporters, with IC_{50} values of 170 μM (L1210) and 166 μM (Walker 256). Propentofylline was a weak inhibitor of *cif* transport, with an IC_{50} value of 6 mM. If propentofylline is a competitive inhibitor of these processes, then these IC_{50} values are representative of K_i values since the concentration (1 μM) of [^{3}H]adenosine used in these experiments was considerably lower than the reported K_m values for adenosine of 16, 19, 15 and 12 μM for *es*, *ei*, *cif* and *cit* transporters, respectively [17, 18, 23, 41]. An adenosine concentration of 1 μM was chosen in the concentration–effect assays for propentofylline potency because concentrations of this magnitude have been observed *in vivo* in ischemic brain [3, 4, 42].

The plasma concentrations of propentofylline in gerbils, rats, and cats that are associated with neuroprotection range from 1 to 50 μM.* The apparent affinity of propentofylline for the *es* transporter (IC_{50}, 9 μM) was within the range of pharmacologically achievable concentrations, suggesting that inhibition of *es* transport is important for neuroprotection. Inhibition of *ei* transport occurred at higher concentrations and, unless propentofylline is concentrated in brain, is unlikely to be an important mechanism of neuroprotection. Inhibition of *cif* transport is probably not of therapeutic importance since the concentrations of propentofylline required for inhibition were very high.

Propentofylline inhibited [^{3}H]NBMPR binding to L1210/B23.1 cells with a K_i value of 37 μM. Previously, propentofylline was found to inhibit competitively [^{3}H]NBMPR binding in rat brain tissue sections with a K_i value of 25 μM [14] and in guinea pig cardiac membranes with a K_i value of 270 μM [15]. The 10-fold difference in affinities for [^{3}H]-NBMPR binding sites in mouse and rat relative to guinea pig may be due to the different methods and preparations used. Alternatively, these differences may indicate a species selectivity that does not correspond to the well-characterized species differences in dipyridamole affinity for *es* transporters [28, 32, 33, 43]. Dipyridamole has the highest potency for inhibition of [^{3}H]nucleoside influx and of [^{3}H]NBMPR binding in human and guinea pig (K_i values of 1–20 nM) followed by mouse (K_i values of 0.1–0.5 μM) and is weakest in rat (K_i values of 1–5 μM).

In conclusion, propentofylline enhances extracellular adenosine concentration in ischemic brain [4], provides neuroprotection to ischemic brain [3, 4, 12], and has greatest affinity for the *es* form of nucleoside transport (present work). Our results suggest that inhibition of *es* transport processes are the basis of the effects of propentofylline on extracellular adenosine concentrations in areas of the brain vulnerable to ischemic damage. The mechanism of action of propentofylline appears to be an indirect stimulation of adenosine A_1 receptors resulting from an increase in extracellular adenosine concentrations. Propentofylline and other inhibitors of *es* transport that cross the blood–brain barrier may be useful therapeutic agents for ischemic brain disease.

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