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# BENZODIAZEPINE INHIBITION OF NUCLEOSIDE TRANSPORT IN HUMAN ERYTHROCYTES

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**Abstract**—The interaction of several benzodiazepines (BDZs) with the nucleoside transport system of fresh erythrocytes from humans was investigated. The affinities of BDZs for the nucleoside transport system were estimated by measuring BDZ inhibition of (a) the site-specific binding of nitrobenzylthioinosine, a potent and specific inhibitor of nucleoside transport, and (b) the uridine transport processes, zero-*trans* influx, zero-*trans* efflux, and equilibrium exchange influx. The BDZs inhibited both the inward and outward transport processes, and, for individual agents, inhibition constants ($K_i$) were similar for the inhibition of each transport process and for the inhibition of the site-specific binding of nitrobenzylthioinosine. The order of potencies of the BDZs in their interactions with the nucleoside transport mechanism (Ro 5-4864 > diazepam > clonazepam > oxazepam > lorazepam > flurazepam) is distinct from the potencies of these compounds at BDZ recognition sites. The affinities of the BDZs for the nucleoside transport system, which are about 1000-fold lower than for BDZ recognition sites, suggest that significant inhibition is unlikely to occur with the plasma concentrations (less than 1 $\mu$M) that result from usual anxiolytic doses of these agents.

Diazepam and other benzodiazepines (BDZs) have wide therapeutic application as anxiolytics, anticonvulsants and muscle relaxants. Diazepam is also commonly used in anesthetic practice and has been advocated for the induction of anesthesia. In the CNS, binding of BDZs to specific, high affinity, "neuronal" BDZ recognition sites may facilitate $\gamma$-aminobutyric acid neurotransmission [1]. Interactions of purine nucleosides at BDZ binding sites in the CNS have also been demonstrated. For example, inosine has been proposed as an endogenous ligand for these membrane sites [2, 3], and the central neuronal depressant effects of adenosine are potentiated by diazepam [4, 5]. Potentiation of adenosine action by diazepam has also been demonstrated with cardiac and smooth muscle *in vitro* [6] and coronary blood flow *in vivo* [7].

Earlier reports noted that effects of 2-chloroadenosine as an agonist at adenosine sites were not enhanced by diazepam [6] nor by recognized inhibitors of nucleoside transport [8]. These results are consistent with the idea that potentiation of adenosine effects by diazepam may be attributed to inhibition of the nucleoside transport system with a consequent decrease in cellular uptake and metabolism of adenosine.

Inhibition of adenosine uptake by BDZs has been demonstrated in guinea pig brain slices [9, 10], rat cortical synaptosomes [11], and guinea pig cardiac muscle [12] and attributed to BDZ inhibition of adenosine transport [11]. However, the observed rates of cellular or vesicular uptake of adenosine probably represent the effects of several metabolic processes in addition to that of membrane transport. Changes with time in the cell content of isotope derived from labeled adenosine may reflect entry by passive diffusion, metabolic trapping by conversion of adenosine to impermeable phosphate esters, and efflux of inosine and hypoxanthine. Initial rates of permeant influx represent unidirectional influx and are those of the first step (transport) in the multistep process of adenosine uptake. Thus, it is critical in the interpretation of permeant uptake rates in terms of transport that those rates be initial rates. In a previous study [13], we evaluated indirectly the affinities of several BDZs for the nucleoside transport system of outdated human erythrocytes by studying their inhibitory effects on the site-specific binding of nitrobenzylthioinosine (NBMPR), a potent and specific inhibitor of nucleoside transport [14]. Nucleoside transport activity ceases when specific membrane sites, evidently on the transporter elements, are occupied by NBMPR and certain congeners, or by dipyridamole. NBMPR binds tightly ($K_D$, 0.1 to 1 nM), but reversibly, to these transport inhibitory sites, and inhibition of transport is related to site occupancy [15]. The NBMPR binding sites appear to be present only on functional nucleoside transport elements [16–18].

This report describes a study of the interaction of BDZs with the nucleoside transport system of fresh erythrocytes from humans. Nucleoside transport in this system is a facilitated diffusion process in which purine and pyrimidine nucleosides are substrates. Nucleoside transport activity was evaluated through initial rate kinetics of the uptake of uridine, a non-metabolized permeant in these cells, using rapid sampling techniques. Estimates of the affinities of several BDZs for the transport mechanism, obtained

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by measuring their inhibitory effects on uridine transport and on NBMPR binding, were compared. Also, in order to examine whether BDZs have different affinities for the inward and outward facing aspects of the nucleoside transporter, the inhibition kinetics of zero-*trans* influx and zero-*trans* efflux of uridine were determined, in addition to the inhibition kinetics of uridine equilibrium exchange.

## MATERIALS AND METHODS

*Erythrocytes.* Blood (5–10 ml) from healthy human volunteers was collected into heparinized (Vacutainer) tubes and centrifuged at 1000 *g* for 10 min. The plasma and buffy coats were discarded, and the erythrocytes were washed three times with 20 vol. of Dulbecco's [19] phosphate buffered saline (PBS). Red cell counts and hematocrit estimations were performed by established methods [20].

*NBMPR binding.* Assays of the site-specific binding of NBMPR to cells were conducted in polypropylene microcentrifuge tubes (1.5 ml) at 22°. Assay mixtures (containing $1.5 \times 10^7$ cells in a final volume of 1.0 ml) were completed by the addition of cell suspension to appropriate concentrations of [G-$^3$H]NBMPR, then incubated for 20 min, and finally centrifuged in an Eppendorf microcentrifuge (12,000 *g*, 10 sec). Cell pellets were washed once with ice-cold PBS (1 ml) and dissolved in 0.5 N KOH (250 μl) prior to assay of $^3$H-radioactivity by liquid scintillation spectrometry. Nonspecific binding of [G-$^3$H]NBMPR, which was less than 10% of the total binding, was defined as the NBMPR which remained cell-associated when assay mixtures contained nitrobenzylthioguanosine (NBTGR) (10 μM) or dipyridamole (10 μM). Under these conditions, the transport inhibitors, compounds which are tightly bound to the nucleoside transport mechanism, displaced all site-bound NBMPR. NBMPR binding constants ($K_D$ and $B_{max}$) were determined by mass law analysis. To determine inhibition constants ($K_i$), cells were incubated in medium containing NBMPR (0.2 to 0.8 nM) in the absence and in the presence of two or three concentrations of each inhibitor; binding data from these experiments were evaluated by mass law analysis. The $IC_{50}$ values (concentration of inhibitor required to inhibit binding by 50%) for the inhibition of NBMPR binding by various compounds were determined by fitting simple linear regression lines to plots of site-specific binding of NBMPR (initial concentration 0.35 nM) against inhibitor concentration.

*Nucleoside transport.* All assays were performed at 22°. Intervals of nucleoside uptake were initiated by rapidly mixing equal volumes of cell suspension and incubation medium and were terminated by an "inhibitor-oil-stop" method. In the stopping method, portions of the assay mixture were transferred to microcentrifuge tubes containing ice-cold stopping medium (5 μM NBTGR in PBS) layered on ice-cold *n*-dibutylphthalate. The tubes were immediately centrifuged at 12,000 *g* for 10 sec. The aqueous and *n*-dibutylphthalate layers were aspirated, and the insides of the tubes were wiped dry. Cell pellets were lysed with 0.5 ml of 0.5% Triton X-100 (v/v), and the samples were deproteinized with 0.5 ml of 5% trichloroacetic acid (v/v). The samples were then centrifuged and portions of the supernatant fractions were retained for estimation of $^{14}$C-radioactivity by liquid scintillation spectrometry. Transport rates were calculated after subtraction of $^{14}$C-activity which became cell-associated due to non-mediated permeation and to equilibration with the extracellular space of the pellet; these corrections were obtained in each experimental protocol by performing parallel experiments in the presence of NBTGR (5 μM) at 0°.

*Equilibrium exchange influx.* Erythrocytes were "loaded" with uridine (0.125 to 10 mM) by incubation with uridine-containing PBS for 2 hr, by which time equilibrium between intracellular and extracellular uridine had occurred. In assays of equilibrium exchange diffusion, to initiate the process 0.2 ml portions of "loaded" cell suspension were rapidly mixed with PBS (final hematocrit 10%) containing the same concentration of [U-$^{14}$C]uridine and, where appropriate, inhibitor. Intervals of permeant uptake (3–5 sec) were chosen such that initial rates of equilibrium exchange influx were obtained.

*Zero-*trans *influx.* Rates of zero-*trans* influx of uridine were determined by incubating erythrocytes (final hematocrit 10%) in [U-$^{14}$C]uridine (0.1 to 1.0 mM). Rates of permeant influx were constant (and representative of initial rates) during the intervals of permeant uptake (3–5 sec) employed in assays of zero-*trans* influx (Fig. 3). In inhibition studies, test compounds and [U-$^{14}$C]uridine were added simultaneously.

*Zero-*trans *efflux.* Erythrocytes were equilibrated with [U-$^{14}$C]uridine (0.1 to 1.0 mM), as in the equilibrium exchange experiments. The "loaded" cells were recovered by centrifugation, and PBS (0.4 ml), with or without inhibitor, was immediately added to the cell pellet with vigorous mixing (final hematocrit 5%). Efflux of [U-$^{14}$C]uridine was assayed by following either the appearance of $^{14}$C-radioactivity in the medium or the disappearance of $^{14}$C-radioactivity from the cell pellet following termination of efflux by the inhibitor-oil-stop method. Intervals of permeant efflux (3–5 sec) were chosen such that the intracellular concentrations of [U-$^{14}$C]uridine did not decrease by more than 30% of the initial value in most instances (Fig. 3).

*Chemicals and drugs.* [G-$^3$H]Nitrobenzylthioinosine (16 Ci/mmole) was purchased from Moravek Biochemicals Inc. (Brea, CA) and [U-$^{14}$C]uridine (529 mCi/mmole) was obtained from Amersham (Oakville, Canada). Where necessary, these radiochemicals were purified to greater than 98% radiochemical purity by high performance liquid chromatography using a $C_{18}$ μBondapak column (Waters) eluted with methanol–water solutions. Nitrobenzylthioinosine and nitrobenzylthioguanosine were synthesized [21] from 6-thioinosine and 6-thioguanosine, respectively, provided by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Dipyridamole was a gift from Boehringer Ingelheim (Canada) Ltd. (Burlington, Canada). Lorazepam and oxazepam were donated by Wyeth Ltd. (Windsor, Canada), and all other benzodiazepines were donated by Hoffman La Roche (Canada) Ltd. (Vaudreuil, Canada).

## RESULTS

*NBMPR binding.* As others have observed [15, 16], the NBMPR associated at equilibrium with human erythrocytes consisted of two components, one of which was site-specific and saturable; the cell content of the other NBMPR component was proportional to free NBMPR concentration (Fig. 1). Mass law analysis of site-specific binding data (Fig. 1 inset) indicated the existence of a single class of sites at which the dissociation constant for NBMPR was 0.31 ± 0.02* nM and the maximum number of binding sites per erythrocyte was 11,000 ± 600 (9)*.

*BDZ inhibition of NBMPR binding.* The benzodiazepines, Ro 5-4864, diazepam, clonazepam and lorazepam, competitively inhibited the binding of NBMPR to fresh human erythrocytes with $K_i$ values of 2.2 ± 0.6 (6), 6.8 ± 1.1 (6), 24.1 ± 4.8 (6) and 45.1 ± 6.9 (6) μM, respectively, as determined by mass law analysis using the double-reciprocal plot method (Fig. 2 and Table 1). The interaction of BDZs with the NBMPR binding site appears to be influenced by the conformation at one BDZ chiral centre because the affinity for the site of the (−)-isomer, Ro 11-6893 ($IC_{50}$ = 38.5 μM), was found to be higher than that of the (+)-isomer, Ro 11-6896 ($IC_{50}$ = 277.3 μM) (Table 2). As shown previously [13], several other BDZs also inhibit NBMPR binding to human erythrocytes, but with an order of potency completely different from that found at the CNS binding site for BDZs [23]. Flurazepam, one of the most potent BDZs at the latter sites, was shown previously [13] to be a poor inhibitor of NBMPR binding, and we now report that the active

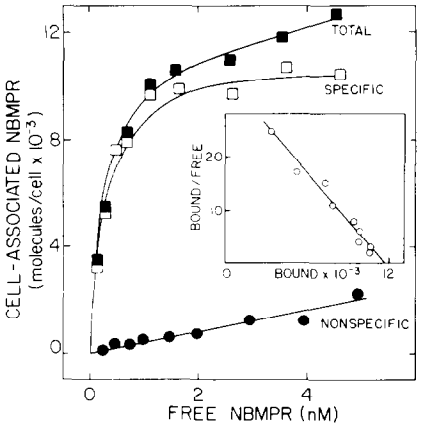


Fig. 1. Binding of NBMPR to human erythrocytes. Erythrocytes were incubated with graded concentrations of NBMPR in the absence (total binding) and presence (non-specific binding) of 10 μM NBTGR for 20 min at 22°. NBMPR concentrations represent free concentrations at equilibrium. Inset: Mass law analysis (Scatchard plot) of the relationship between specific binding of [$^3$H]NBMPR and the equilibrium concentrations of free [$^3$H]NBMPR. Ordinate: ratio of bound NBMPR to free NBMPR (molecules/cell × $pM^{-1}$); abscissa: bound NBMPR (molecules/cell × $10^{-3}$).

* Throughout this report, mean values ± S.E. are cited and are followed by a number in parentheses representing the number of replicate assays.

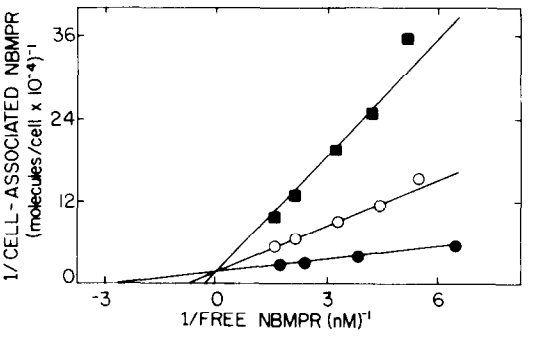


Fig. 2. Mass law analysis of diazepam (●, 0; ○, 30; and ■, 100 μM) inhibition of the binding of NBMPR to human erythrocytes. Each concentration of NBMPR has been corrected for ligand depletion, and the points shown are the equilibrium concentrations of free ligand in the medium. The plot indicates diazepam to be a competitive inhibitor of the binding of NBMPR with a $K_i$ of 7 μM.

flurazepam metabolite, didesethylflurazepam, was also a weak inhibitor of NBMPR binding (Table 2). The BDZ antagonist, Ro 15-1788 (20% inhibition at 300 μM), was also a weak inhibitor of NBMPR binding (Table 2) and the water-soluble BDZ, midazolam, was weaker than diazepam with an $IC_{50}$ of 51.3 μM (Table 2). Specificity of the inhibition of NBMPR binding was tested by examining the effect of various other non-BDZ compounds on the binding of NBMPR. Compounds tested at 300 μM for their inhibition of NBMPR binding were hydrocortisone, corticosterone, pancuronium, tubocurarine, quinidine, morphine, fentanyl, ouabain, cocaine, imipramine, phentolamine, histamine, cimetidine, mepyramine, and phenytoin. Of these, only corticosterone, quinidine, phenytoin and pancuronium significantly inhibited NBMPR binding, producing 68 ± 2, 39 ± 6, 16 ± 3 and 15 ± 6% inhibition at 300 μM respectively.

*Nucleoside transport.* The equilibrium exchange influx of uridine into human erythrocytes was a rapid, saturable process at 22° (Fig. 3). The kinetic parameters observed in such experiments were: $K_m$, 0.76 ± 0.07 (6) mM, and $V_{max}$, 328 ± 42 (6) mmoles per l cells per hr. The kinetic constants for the zero-*trans* influx of uridine were: $K_m$, 0.17 ± 0.02 (3) mM, and $V_{max}$, 82 ± 10 (10) mmoles per l cells per hr. For the zero-*trans* efflux of uridine in these cells, the kinetic constants were: $K_m$, 0.14 ± 0.02 (4) mM, and $V_{max}$, 96 ± 11 (4) mmoles per l cells per hr. These values differ from those reported previously using erythrocytes from outdated stored blood [24].

*BDZ inhibiton of nucleoside transport.* In preliminary experiments, it was shown that the extent of BDZ inhibition of uridine influx (extracellular concentration, 1 mM) was not enhanced by preincubating erythrocytes for 30 min at 22° with BDZs. Thus, inhibitions of 41, 65, 35 and 37% were obtained upon simultaneous addition of uridine with diazepam (10 μM), Ro 5-4864 (10 μM), clonazepam (50 μM) and lorazepam (75 μM), respectively; these values were not significantly different from inhibitions of 49, 71, 40 and 38% obtained when cells were preincubated with these BDZs (same order and con-

Table 1. Comparison of benzodiazepine inhibition constants

| | Inhibition of NBMPR binding to hRBC* $K_i$ ($\mu$M) | Inhibition of equilibrium exchange uridine influx in hRBC* $K_i$ ($\mu$M) | Inhibition of diazepam binding to guinea pig heart tissue† $K_i$ ($\mu$M) |
|---|---|---|---|
| Ro 5-4864 | 2.2 | 8 | 0.0022 |
| Diazepam | 6.8 | 11 | 0.048 |
| Clonazepam | 24.1 | 40 | 2.3 |
| Lorazepam | 45.1 | 83 | |

* Inhibition constants ($K_i$) for the BDZ-induced inhibition of the site-specific binding of NBMPR and equilibrium exchange uridine influx were estimated as described in the text.

† Data for BDZ-induced inhibition of diazepam binding to guinea pig cardiac membranes are from Ref. 22.

centrations). Accordingly, later experiments were performed without preincubating cells with the BDZs.

The inhibition of equilibrium exchange influx of uridine by the BDZs was apparently competitive, with order of potency and $K_i$ values similar to those found for the BDZ inhibition of NBMPR binding (Fig. 4 and Table 1). Diazepam and lorazepam inhibited zero-*trans* influx and zero-*trans* efflux of uridine in erythrocytes with $K_i$ values of 8.4 and 91 $\mu$M for inhibition of influx, and $K_i$ values of 11.4 and 64 $\mu$M for inhibition of efflux respectively (Fig. 5). These values are similar to the $K_i$ values determined for their inhibition of the equilibrium exchange influx of uridine; the $K_i$ value of diazepam inhibition of that process was 11 $\mu$M and that for lorazepam inhibition was 83 $\mu$M (Table 1). However, in contrast to their apparently competitive inhibition of equilibrium exchange influx, the BDZs appeared to be noncompetitive inhibitors of zero-*trans* uridine influx and zero-*trans* uridine efflux.

The BDZs flunitrazepam, oxazepam and flurazepam also inhibited the zero-*trans* influx of uridine (initial extracellular concentration 1 mM) with $IC_{50}$ values of 15, 46, and 70 $\mu$M respectively. Dimethyl sulfoxide was added as indicated to solubilize BDZs, but it did not affect uridine transport in concentrations up to 1%.

Table 2. Inhibition of the site-specific binding of NBMPR to hRBC*

| | $IC_{50}$ ($\mu$M) | % Inhibition at 300 $\mu$M |
|---|---|---|
| Midazolam | 51 ± 2 | 90 ± 1 |
| Ro 11-6893(−) | 39 ± 3 | 95 ± 1 |
| Ro 11-6896(−) | 277 ± 9 | 51 ± 2 |
| Didesethylflurazepam | | 22 ± 2 |
| Ro 15-1788 | | 20 ± 5 |

* Estimations of the site-specific binding of NBMPR (initial concentration of 0.35 nM) were performed as described in the text. Each inhibitor was tested at a minimum of six different concentrations and $IC_{50}$ values are the means (± S.E.) from at least three experiments performed in duplicate. For the compounds which produced less than 50% inhibition at the highest concentration tested (300 $\mu$M), only percent inhibition values are given.

## DISCUSSION

This study demonstrated that BDZs inhibited nucleoside transport in fresh human erythrocytes.

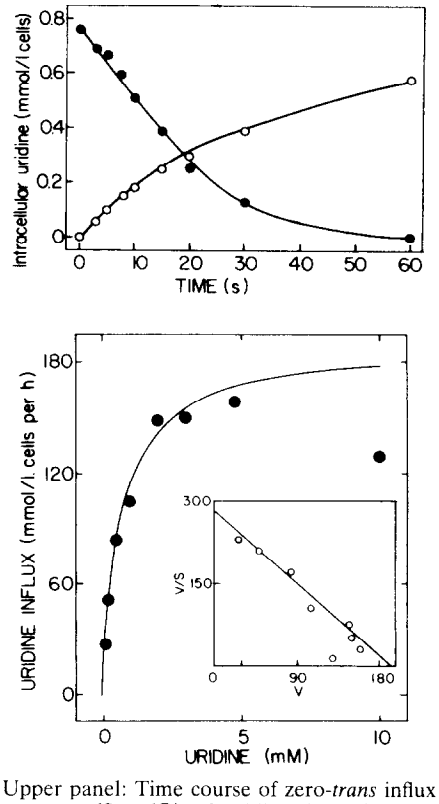


Fig. 3. Upper panel: Time course of zero-*trans* influx (○) and zero-*trans* efflux (●) of uridine (1 mM) in human erythrocytes at 22°. Intervals of permeant influx were initiated as described in Materials and Methods and terminated by the inhibitor-oil-stop method. For subsequent experiments, intervals of permeant flux (3–5 sec) were chosen in order that initial rates (which are representative of transport rates) were measured. Lower panel: Effect of the extracellular concentration of uridine on uridine influx in equilibrium exchange in human erythrocytes. The curve describing the influx of uridine was calculated from the equation

$$v \text{ (mmoles/l cells/hr)} = V_{max} \cdot (s)/(K_m + (s))$$

where $v$ is the velocity of influx at an extracellular concentration of $s$ mM. The kinetic constants, $K_m$ and $V_{max}$, were calculated from a least squares analysis of either a $v/s$ vs $v$ plot of the data (inset) or a $s/v$ vs $s$ plot.

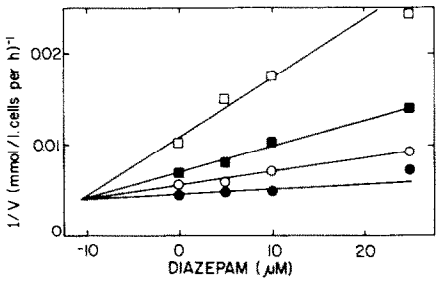


Fig. 4. Dixon plot of diazepam inhibition of equilibrium exchange influx of uridine (●, 5; ○, 2; ■, 1; and □, 0.5 mM) in human erythrocytes. [U-$^{14}$C]Uridine and the appropriate concentration of diazepam were added together to cell suspensions which had been incubated in nonradioactive uridine. The plot indicates diazepam to be a competitive inhibitor with a $K_i$ of 11 μM.

$K_i$ values for BDZ inhibition of the uridine transport processes, zero-*trans* influx, zero-*trans* efflux and equilibrium exchange influx, were equivalent and similar to those estimated for BDZ inhibition of the binding of NBMPR, a potent and specific inhibitor of nucleoside transport. Dipyridamole is also a potent inhibitor of nucleoside transport, having a potency about 1000-fold greater than that of BDZs. We have found that the $K_i$ values for the dipyridamole inhibition of uridine entry and exit, and for NBMPR binding, were also similar (J. R. Hammond, S. M. Jarvis, A. R. P. Paterson and A. S. Clanachan, unpublished data).

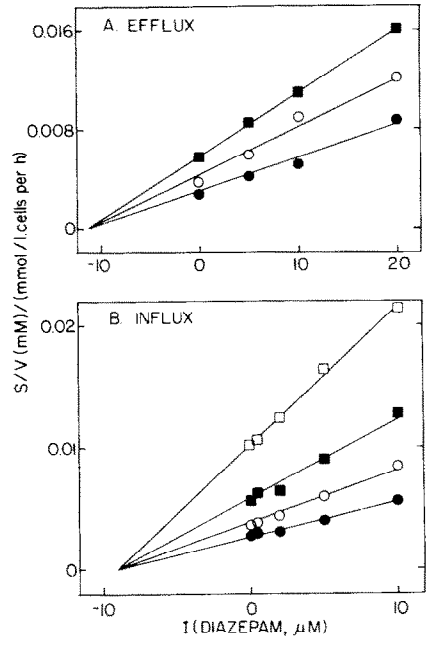


Fig. 5. Inhibition of zero-*trans* efflux (A) and zero-*trans* influx (B) of uridine in human erythrocytes by diazepam. Data are presented as an $s/v$ vs $I$ plot where $s$ is either the intracellular (A) or the extracellular (B) [U-$^{14}$C]uridine concentration (mM) and $v$ is the initial rate of uridine flux (mmoles/l cells/hr). The intracellular concentrations of uridine in efflux experiments (A) were 0.5 (■), 0.2 (○) and 0.1 (●) mM, and the extracellular concentrations in influx experiments (B) were 1.0 (□), 0.49 (■), 0.24 (○) and 0.13 (●) mM. See text for other experimental details. Diazepam inhibited the efflux and influx of uridine in a noncompetitive manner with $K_i$ values of 11.4 and 8.4 μM respectively.

Inhibition of nucleoside transport, which might be expected to potentiate the extracellular effects of adenosine and other nucleosides, has been invoked in postulates about the central [11] and peripheral [6] actions of diazepam and other BDZs. Several studies have indicated that BDZs inhibit the accumulation of adenosine in brain tissue [9, 10]. However, those studies did not show whether or not inhibition of the multistep process of cellular accumulation of adenosine occurred at the level of nucleoside metabolism, or at the level of nucleoside transport, the first step in the uptake process. Because rates of nucleoside transport, in some instances, exceed rates at which internalized nucleoside molecules are enzymatically transformed, time courses for cellular uptake of nucleosides are complex. This has necessitated use of rapid sampling techniques to obtain the definitive time courses of cellular nucleoside uptake which are needed to obtain initial rates of nucleoside uptake; the latter measure transport rates. Thus, interpretation of cellular nucleoside uptake rates as transport rates (and of inhibition of nucleoside uptake as transport inhibition) requires that the uptake rates be demonstrably initial rates. To study uridine permeation in human erythrocytes in this way, intervals of uptake must be brief (1–5 sec). The present study meets these criteria for defining the transport of uridine [25] and demonstrates that BDZs inhibit both the inward and outward transport processes.

We have shown elsewhere [26] that, while the kinetic symmetry of the nucleoside transport system differs between fresh and stored erythrocytes, both possess similar numbers of NBMPR binding sites. These sites on fresh cells also display a slightly higher affinity ($K_D$ = 0.31 nM) for NBMPR than do stored cells ($K_D$ = 0.97 nM). The former value is comparable to the affinities of NBMPR binding sites on cultured cells [17, 27] and CNS membranes [28].

It is not yet known whether inhibition of nucleoside transport in erythrocytes by NBMPR derives from binding of the latter at the permeation site or at some other site on the transporter. The inhibition by NBMPR of uridine influx and uridine equilibrium exchange was apparently competitive, as was the inhibition of the site-specific binding of NBMPR to erythrocyte membranes by nucleoside permeants, such as uridine and deoxycytidine, are apparently competitive [29, 30]. These findings are consistent with the notion that NBMPR competes with nucleosides at the permeation site of the transport system [18, 30]. However, the apparent purine specificity of the NBMPR binding sites in HeLa cells [31] is not consistent with the broad permeant specificity of the nucleoside transport system. It is also possible that these kinetic and binding results could be a consequence of inhibitor-induced conformational changes in the transporter. Thus, at the present time, the relationship between the NBMPR binding site and the permeation site is uncertain. Consequently, it

was considered important to compare BDZ inhibition constants derived from measurements of the inhibition of the binding of NBMPR [13] with those obtained via the direct measurement of nucleoside transport. With individual agents, inhibition constants ($K_i$) were similar for inhibition of (i) uridine influx, (ii) uridine efflux, and (iii) site-specific binding of NBMPR. The present study also demonstrated that BDZs interact with the nucleoside transport mechanism of erythrocytes with apparently competitive effects in the inhibition of (i) site-specific binding of NBMPR and (ii) equilibrium exchange (influx) of uridine. However, BDZ-induced inhibition of zero-*trans* influx and zero-*trans* efflux of uridine were of an apparently noncompetitive nature. These results are consistent with the notion that BDZs are capable of binding to both the inward and outward facing conformations of the transport mechanism [32]. Furthermore, according to the criteria of Deves and Krupka [32], these results also suggest that BDZs do not undergo transport on the nucleoside carrier.

The order of the BDZ potencies in their interactions with the nucleoside transport mechanism (Ro 5-4864 > diazepam > clonazepam > oxazepam > lorazepam > flurazepam) as determined by inhibition of (a) uridine transport and (b) NBMPR binding is distinct from the potencies of those compounds at both the neural and non-neuronal BDZ recognition sites (see Table 1). The affinities of BDZs for NBMPR sites on CNS membranes have been reported [28] and are similar to those estimated in the present study. Therefore, the neuronal BDZ binding sites, which may be the receptors at which the BDZs act to produce their CNS effects, are unrelated to the NBMPR sites associated with the nucleoside transport mechanism. The BDZs possess high affinity for the former ($K_i$ of diazepam is 7.4 nM) [23], but low affinity for the latter ($K_i$ for diazepam is 10 $\mu$M) relative to that of NBMPR or dipyridamole. A further distinction between these sites comes from the observation that the NBMPR sites on erythrocytes and CNS membranes display a selectivity for the (−)-isomer of the BDZ stereoisomeric pair Ro 11-6896(+) and Ro 11-6893(−), whereas the neuronal BDZ site prefers the (+)-isomer. In addition, the nucleoside transport inhibitor, dipyridamole, has high affinity ($K_i$ = 11 nM) for the transport site in CNS membranes [28] and low affinity for the BDZ site [33]. NBMPR sites are also unrelated to peripheral BDZ sites (Table 1).

Thus, a type of site exists on the nucleoside transport mechanism at which (a) BDZs have low affinity, and (b) agents related to NBMPR and dipyridamole have high affinity. Similar sites exist on guinea pig CNS membranes [28]. BDZ affinities for these sites correlate with their abilities to inhibit nucleoside uptake in guinea pig cardiac muscle [12] and to potentiate the effects of adenosine in cardiac [6] and smooth muscle [6]. The affinities of BDZs for the nucleoside transport system suggest that significant inhibition is unlikely with the plasma concentrations (less than 1 $\mu$M) that result from usual anxiolytic doses of these agents. Inhibition of nucleoside transport by some BDZs, at the higher plasma concentrations associated with induction of anesthesia, may explain the potentiation of adenosine effects in central neuroeffector systems.

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