0026-895X/86/060659-07$02.00/0

MOLECULAR PHARMACOLOGY, 30:659–665

Nitrobenzylthioinosine-Sensitive Nucleoside Transport System: Mechanism of Inhibition by Dipyridamole

SIMON M. JARVIS

Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom

Received May 12, 1986; Accepted September 15, 1986

SUMMARY

Dipyridamole-mediated inhibition of nucleoside transport by the nitrobenzylthioinosine (NBMPR)-sensitive facilitated diffusion system in mammalian erythrocytes was investigated. [^{3}H]Dipyridamole was a competitive inhibitor of uridine equilibrium exchange influx into guinea pig erythrocytes (apparent K_i 1 nM). Analysis of the results using total inhibitor levels instead of cell-free inhibitor concentrations increased the apparent K_i value to 7 nM. Similarly, [^{3}H]dipyridamole inhibition of zero-*trans*-[^{14}C] uridine influx was consistent with simple competitive inhibition (apparent K_i 1.4 ± 0.7 nM). In contrast, [^{3}H]dipyridamole behaved as a noncompetitive inhibitor of zero-*trans*-[^{14}C]uridine efflux (apparent K_i 0.7 ± 0.2 nM). In a second series of experiments, [^{3}H]dipyridamole was found to bind to a single class of high affinity sites on plasma membranes from human erythrocytes (apparent K_d 0.65 ± 0.07 nM) with a maximum number of binding sites similar to that determined with the nucleoside transport inhibitor NBMPR. Binding of dipyridamole to these sites was blocked by the nucleoside transport inhibitors NBMPR, nitrobenzylthioguanosine, and dilazep and in a competitive manner by adenosine and uridine (apparent inhibition constants 0.1 and 0.9 mM, respectively). These inhibition constants are similar to the apparent K_m for adenosine and uridine equilibrium exchange in human erythrocytes. These results are consistent with the notion that, in mammalian erythrocytes, dipyridamole interacts with the NBMPR-sensitive transporter at the same site as NBMPR, which is preferentially located on the outer surface of the cell membrane totally or partially within the permeation site.

Nucleosides and nucleoside analogues enter most mammalian cells by saturable facilitated diffusion systems that are inhibited by the drug dipyridamole (1–3). Dipyridamole is a vasodilator and an antithrombotic agent which has been used in the treatment of angina and as an antiplatelet drug (4, 5). In more recent studies, it has been shown that dipyridamole can enhance the effectiveness of various antimetabolites, for example, acivicin, 5-fluorouracil, deoxyadenosine, and methotrexate, by inhibiting either nucleic acid precursor salvage and/or perhaps preferentially blocking nucleoside efflux (6–10). There is therefore considerable interest in possibly using dipyridamole in combination chemotherapy with inhibitors of *de novo* nucleotide synthesis. Nevertheless, the mechanism by which dipyridamole inhibits nucleoside flux is in dispute. In influx experiments competitive inhibition by dipyridamole has been demonstrated for dog erythrocytes (11), nucleoside-permeable sheep erythrocytes (12), chick fibroblasts (13), Novikoff rat hepatoma cells (14), and human platelets (15). However, the apparent K_i values range from 0.02 to 47 μM. In other experiments, inhibition of adenosine uptake by human erythrocytes at 1° was of the mixed type (apparent K_i 0.23 μM) (16). Conversely, dipyridamole can displace specific bound NBMPR, a potent specific competitive inhibitor of nucleoside transport (12, 17), from erythrocytes and membranes prepared from brain, heart, lung, and liver and from HeLa and Chinese hamster ovary cells (18–24). In some of these investigations dipyridamole was shown to be a competitive inhibitor of [^{3}H] NBMPR binding (apparent K_i values 0.4–276 nM) and it was suggested that both agents bind to the same site (18, 19, 22–24). In other studies it has been concluded that dipyridamole acts at a site distinct from the NBMPR-binding site (25–27).

A common assumption in the kinetic analysis of both transport and NBMPR binding inhibition by dipyridamole has been that the concentration of free inhibitor in the medium is the same as the total (free + bound) inhibitor concentration of the cell suspension. However, recent experiments have demonstrated this not to be the case for NBMPR inhibition of nucleoside transport in erythrocytes (12). Furthermore, dipyridamole can inhibit the transport of anions, purine bases, 2-deoxy-D-glucose, and D-glucosamine in various cell systems (28, 29) and, thus, these transport sites offer the potential for further depletion of dipyridamole. Also, dipyridamole has high affinity for peripheral benzodiazepine-binding sites (30) and is a highly lipophilic drug. An additional complication is that

During part of this study S. M. J. was a Scholar of the Alberta Heritage Foundation for Medical Research in the Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. This work was supported partially by the Medical Research Council of Canada.

ABBREVIATIONS: NBMPR, nitrobenzylthioinosine; EDTA, ethylenediaminetetraacetate; NBTGR, nitrobenzylthioguanosine.

nucleoside transport in rat tissues is approximately 2–3 orders of magnitude less susceptible to inhibition by dipyridamole than corresponding tissues from guinea pig and several other mammalian species (20–22, 31, 32). Thus, the apparent K_i measured, and possibly the inhibition pattern observed, will depend on the experimental conditions and the species employed.

The aim of the present study was to investigate the potency and mechanism of interaction of dipyridamole with the NBMPR-sensitive nucleoside transporter. Two approaches were used. In the first, the effects of dipyridamole on uridine zero-*trans* influx, zero-*trans* efflux, and equilibrium exchange by guinea pig erythrocytes was studied. Guinea pig erythrocytes were chosen as these cells transport nucleosides slowly by a route that can be blocked by low concentrations of NBMPR and dipyridamole (33, 34), and, thus, transport parameters can be accurately determined. Uridine is a well defined and non-metabolized substrate for the carrier (33). In these experiments, [^{3}H]dipyridamole was used to directly measure the free concentration of inhibitor. The second approach was to study the binding of [^{3}H]dipyridamole to human erythrocyte membranes and the ability of nucleosides to inhibit [^{3}H]dipyridamole binding. The present results suggest that dipyridamole interacts with the nucleoside transporter at the same site as NBMPR, which is totally or partially within the outward facing conformation of the permeation site. A preliminary report of some of this work was presented at the 1986 Winter Meeting of the Canadian Physiological Society (for abstract see Ref. 35).

Materials and Methods

Erythrocytes and membrane preparation. Whole blood from female guinea pigs (250–400 g) and healthy human volunteers was collected into heparinized evacuated tubes. Erythrocytes were washed three times with 20 volumes of iso-osmotic NaCl medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl (pH 7.4 at 22°), 2 mM $MgCl_2$, 0.1 mM EDTA (disodium salt), and 5 mM glucose. The buffy coat was discarded. Hemoglobin-free erythrocyte membranes were prepared by osmotic lysis and resuspended in 5 mM sodium phosphate (pH 7.9 at 22°) (36).

Transport measurements. Initial rates of zero-*trans* uridine influx into guinea pig erythrocytes were determined by a washing method using ice-cold iso-osmotic NaCl medium containing 10 μM NBMPR, with the following modifications. The details of this technique have been published previously (12, 33). For the inhibitor studies, [^{3}H]dipyridamole (0.2 ml) was added to the cells (0.1 ml) at the same time as [U-^{14}C]uridine (0.1 ml; 6 μCi/ml). Transport of radiolabeled uridine was terminated at predetermined time intervals (10 min) by rapid centrifugation (12,000 × *g* for 5 sec using a Fisher microcentrifuge). Immediately, aliquots (0.2 ml) of the supernatant were removed in order to measure the equilibrium free concentration of [^{3}H]dipyridamole in the medium at the end of the incubation period. The cell pellets were then rapidly washed with the ice-cold NBMPR stopping solution (12,33). The incubation time chosen was based on previous results (33) that had demonstrated that initial rates of uridine influx were measured using such time intervals. The washed cell pellets were lysed with 0.5 ml of 0.5% (v/v) Triton X-100. Samples were deproteinized by the addition of 0.5 ml of trichloroacetic acid (50 g/liter) and centrifuged (2 min, 12,000 × *g*) to remove the precipitate. Radioactivity in aliquots of the protein-free supernatants was determined by scintillation counting with appropriate quench correction. Blank samples were obtained by processing cell samples that had been mixed with [^{14}C]uridine in the presence of 10 μM NBMPR. Transport rates were calculated after subtraction of these blanks. For inward equilibrium exchange measurements, guinea pig erythrocytes were "loaded" with uridine for sufficient time to allow for equilibration of the uridine. Briefly, equal volumes (2 ml) of cells (20% hematocrit) and uridine were incubated at 37° for 6 hr and then allowed to cool to 22°. Aliquots of these cells (0.2 ml) were then added to solutions (0.2 ml) containing the same concentration of [^{14}C]uridine and, where appropriate, [^{3}H]dipyridamole, and the initial rate of equilibrium exchange uridine influx and the effect of dipyridamole on this flux were determined as described above for the zero-*trans* influx measurements.

For efflux experiments, guinea pig erythrocytes were "preloaded" with various concentrations of [^{14}C]uridine by mixing equal volumes (0.6 ml) of cells (40% hematocrit) and [^{14}C]uridine (6 μCi/ml) at 37° and allowing the nucleoside to equilibrate across the cell membrane. Extracellular isotope was removed by rapidly washing the cells with three 1-ml portions of ice-cold medium, and portions (0.1 ml) of the resulting cell suspension (1.2 ml) were centrifuged. The supernatant was removed and, immediately, 0.9 ml of prewarmed medium at 22° containing varying concentrations of [^{3}H]dipyridamole or 10 μM NBMPR was added to the cell pellet. The initial rate of uridine efflux was measured by following the appearance of ^{14}C radioactivity in the medium. Incubations (10 min) were terminated by layering 0.9 ml of the incubation mixtures on 0.5 ml of ice-cold *n*-dibutyl phthalate in microcentrifuge tubes. The tubes were immediately centrifuged at 12,000 × *g* for 15 sec. Portions (0.7 ml) of the upper layer of cell-free aqueous medium were transferred to 10 ml of scintillation fluid and counted for ^{14}C/^{3}H radioactivity with appropriate quench correction. Previous control experiments (33) confirmed that no significant metabolism of [^{14}C]uridine occurred under these experimental conditions.

Dipyridamole and NBMPR binding assays. Equilibrium NBMPR and dipyridamole binding to membranes prepared from human erythrocytes was determined at 22° based on methods previously described for NBMPR binding to ghosts (12, 36). Briefly, membrane suspensions (40 μg of protein; 50 μl) were added to 1.0 ml of medium (5 mM sodium phosphate, pH 7.9) containing radioactive ligand in the presence and absence of NBTGR (1 μM). NBTGR eliminated site-specific binding of dipyridamole (see Fig. 3). In the case of competition experiments the medium also contained the test compound. After 30 min, incubations were terminated by centrifugation at 15,000 × *g* for 25 min at 4°. Supernatants were retained for radioactivity determinations. Membrane pellets were washed once with ice-cold sodium phosphate medium and ^{3}H radioactivity associated with the pellet was measured by liquid scintillation as previously described (12). Binding constants were determined by mass law analysis of equilibrium binding data after subtraction of nonspecific binding.

Materials. [U-^{14}C]Uridine (specific radioactivity 0.45 Ci/mmol) was obtained from Amersham, Oakville, Ontario. [G-^{3}H]NBMPR (specific radioactivity 20 Ci/mmol) and [^{3}H]Persantin [dipyridamole; 2,2^1,2^{11},2^{111}-(4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine-2,6-diyldinitrilo)tetraethanol] (specific radioactivity 110 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA. The radiochemical purity of [^{3}H]dipyridamole was >98% and was routinely checked during storage at −20° using high performance liquid chromatography. When necessary, [^{3}H]dipyridamole was repurified by high performance liquid chromatography on a C_{18} μBondapak column (Waters Associates, Milford, MA) eluted with 70% methanol/30% water and 0.2% ammonia. After storage for 1 year at −20°, 92% of the radioactivity recovered from the C_{18} column co-eluted with the dipyridamole peak. NBMPR, NBTGR, and dilazep (1,4-bio[3-(3,4,5-trimethoxybenzoyloxy)propyl]perhydro-1,4-diazepine) were generous gifts from Professor A. R. P. Paterson, Cancer Research Group, University of Alberta, Edmonton, and Hoffmann-LaRoche and Co., Basel, Switzerland, respectively.

Results

Dipyridamole inhibition of uridine transport. Previous studies (33) have demonstrated that nucleoside transport by guinea pig erythrocytes is best described by two components: a saturable uptake mechanism that is inhibited competitively by

nM concentrations of NBMPR (17) and a low affinity, nonsaturable uptake mechanism that is not blocked by NBMPR. This NBMPR-insensitive component of nucleoside transport, estimated in the presence of excess nonradioactive NBMPR (10 μM), was subtracted from the total flux data described in this section before kinetic analysis was performed. Control experiments established that the magnitude of this linear NBMPR-insensitive flux was similar to that measured in the presence of high concentrations of dipyridamole (10 μM) (data not shown).

To investigate the kinetics of dipyridamole inhibition of nucleoside transport, the effect of varying concentrations of [^{3}H]dipyridamole (initial concentration 1.25–10 nM) on equilibrium exchange uridine influx (0.05–1.0 mM) by guinea pig erythrocytes was studied at 22°. [^{3}H]Dipyridamole and [^{14}C]uridine were added to cells simultaneously. Fig. 1 shows the results of one such experiment, where reciprocals of the initial rates of uridine equilibrium exchange influx are plotted against the free [^{3}H]dipyridamole concentration. The Dixon plot (37) ($1/v$ versus I) is consistent with simple competitive inhibition (apparent K_i 1.0 nM). Analysis of these data by the Cornish-Bowden method (s/v versus I) (38) also resulted in a simple competitive plot. When inhibitor depletion was not considered, the apparent K_i value determined from the Dixon plot and using the total inhibitor concentration was increased to a value of approximately 7.0 nM.

In other experiments, the effect of [^{3}H]dipyridamole on zero-*trans* uridine influx and efflux was compared. The results from a representative experiment are shown in Fig. 2, where initial rates of [^{14}C]uridine influx and efflux are plotted as s/v against the free [^{3}H]dipyridamole concentration. The parallel lines of the Cornish-Bowden plot (38) for the influx data are indicative

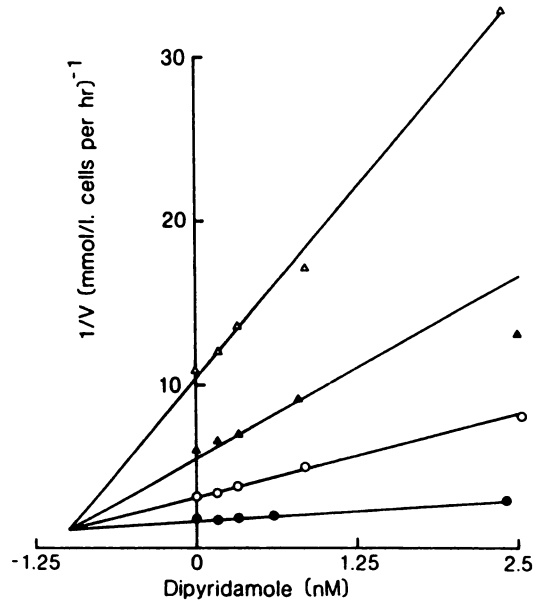

Fig. 1. Effect of [^{3}H]dipyridamole on equilibrium-exchange influx of [^{14}C]uridine by guinea pig erythrocytes. Guinea pig erythrocytes were preincubated with increasing concentrations of uridine (0.05–1.0 mM) and, following equilibrium, [^{14}C]uridine and [^{3}H]dipyridamole were added to cells simultaneously. The initial equilibrium-exchange uridine influx rates at 22° (corrected for the NBMPR-insensitive component) are plotted as $1/v$ versus I, where v is the initial rate of [^{14}C]uridine influx, and I is the final free [^{3}H]dipyridamole concentration. Uridine concentrations (mM) were: 1.0 (●), 0.25 (○), 0.10 (▲), and 0.05 (△). See the text for other experimental details.

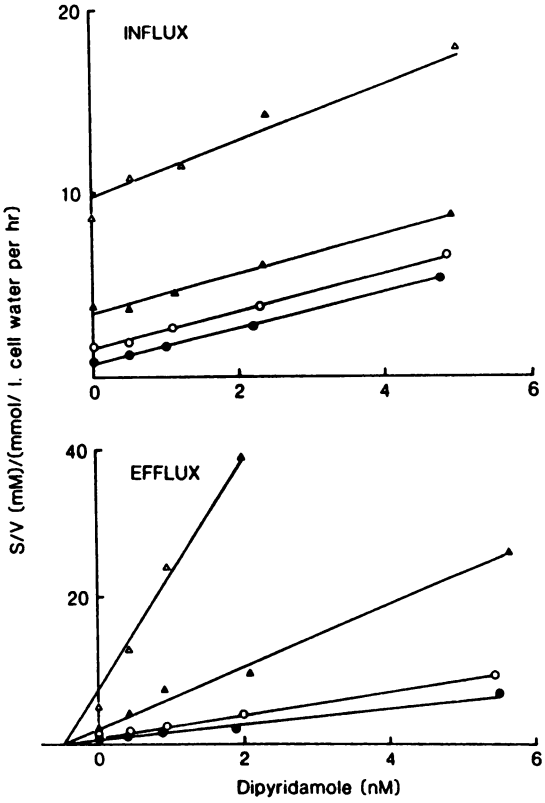

Fig. 2. Effect of [^{3}H]dipyridamole on uridine influx and efflux by guinea pig erythrocytes. The initial rates of uridine influx and efflux were determined as described in Materials and Methods. The flux rates at 22° (corrected for the NBMPR-insensitive component) are plotted as s/v versus I, where s and I are the [^{14}C]uridine and final free [^{3}H]dipyridamole concentrations, respectively, and v is the initial rate of uridine influx. Uridine concentrations (mM) for the influx experiment were: 1.0 (△), 0.25 (▲), 0.1 (○), and 0.05 (●). Initial intracellular uridine concentrations (mmol/liter of cell water) for the efflux experiment were: 1.0 (△), 0.31 (▲), 0.054 (○), and 0.026 (●).

of [^{3}H]dipyridamole acting as a competitive inhibitor. Similarly, competitive inhibition plots were obtained when the data were analyzed by the Dixon procedure (37) (apparent K_i 0.75 nM). In contrast with the effect of [^{3}H]dipyridamole on uridine influx, the lines of the Cornish-Bowden plot (38) for the efflux data intersect on the I axis, consistent with noncompetitive inhibition of zero-*trans* uridine efflux (apparent K_i 0.5 nM). In two other independent experiments, similar inhibition plots were obtained with apparent K_i values of 1.1 and 2.4 nM, and 1.0 and 0.6 nM for influx and efflux, respectively. In a control experiment, the time course of total [^{3}H]dipyridamole association with guinea pig erythrocytes was studied at an initial extracellular inhibitor concentration of 4 nM. Within 1 min, [^{3}H]dipyridamole association with the cells had reached a steady state value of 66 nmol/liter of cells which was approximately 50-fold greater than the final extracellular concentration of dipyridamole. When similar studies were performed with unsealed membranes at the equivalent cell density, [^{3}H]dipyridamole association with the membranes was reduced 5-fold compared to intact cells. This result would suggest that dipyridamole can penetrate erythrocyte membranes rapidly and thus have access to both membrane surfaces.

Dipyridamole binding studies. An alternative method to study the interaction of dipyridamole with the nucleoside trans-

port system is to investigate the binding properties of the inhibitor to plasma membranes. This has only become possible in the last year with the availability of high specific radioactive dipyridamole. Isolated membranes were chosen in preference to intact cells for these experiments because previous studies with another nucleoside transport inhibitor, NBMPR, had demonstrated that the nonsaturable component of NBMPR binding is very much reduced in membranes compared with cells (36, 39). Preliminary studies with membranes prepared from guinea pig erythrocytes showed no detectable high affinity saturable [^{3}H]dipyridamole binding but, instead, a nonsaturable component responsible for the binding of 0.2 pmol/mg of protein at 1 nM (data not shown). NBMPR (10 μM) had no effect on this nonsaturable association. In contrast with [^{3}H]dipyridamole, however, [^{3}H]NBMPR binding to guinea pig ghosts revealed two components of binding: 1) a saturable component with a B_{max} value of 0.048 pmol/mg of protein and an apparent K_d of 73 pM, and a nonsaturable component responsible for the binding of 0.07 pmol/mg of protein at 1 nM. Thus, the high level of nonspecific binding with [^{3}H]dipyridamole made it impossible to detect the low number of nucleoside transporters in guinea pig erythrocytes with this ligand. Therefore, for further experiments, membranes prepared from human erythrocytes were used because these cells have an approximately 500-fold higher number of transport sites, as estimated by NBMPR binding, than guinea pig erythrocytes (40). The increased number of NBMPR-binding sites is directly proportional to the increase in maximum velocity of transport observed in human red blood cells, but in all other respects, the kinetic parameters of nucleoside transport by human and guinea pig erythrocytes are similar (33, 40, 41).

The binding of [^{3}H]dipyridamole to human erythrocyte membranes is shown in Fig. 3, where membrane-associated binding is plotted against the equilibrium free concentration of inhibitor in the medium. The data can be resolved into two components of binding: a saturable association responsible for the binding of 22.7 ± 0.7 pmol/mg of protein with an apparent K_d of 0.52 ± 0.07 nM, and a nonsaturable component responsible for the binding of 0.44 pmol/mg of protein at 1 nM. The saturable association was abolished in the presence of 1 μM NBTGR. Mass law analysis of the saturable high affinity binding components (Fig. 3B) indicated the existence of a single class of dipyridamole-binding sites within the concentration range 0–10 nM. Membranes from four individual donors gave a mean apparent K_d (±SE) of 0.65 ± 0.07 nM with a maximum binding of 29.7 ± 2.8 pmol/mg of protein for the saturable component. The same membrane preparations had a maximum number of site-specific NBMPR-binding sites of 27.2 ± 5.7 pmol/mg of protein, a value not significantly different from that determined using [^{3}H]dipyridamole.

It was necessary to establish that no high affinity dipyridamole-binding activity was lost during the washing procedure adopted to remove unbound radioactivity from the membranes. The differences between supernatant radioactivity counts in the presence and absence of NBTGR were used as an indirect estimate of the maximum high affinity binding activity and gave an estimate of 29.7 ± 4.6 pmol/mg of protein. These binding estimates were similar to those obtained with membrane pellets after washing, demonstrating that no significant loss of binding activity had occurred with washing. In contrast, the magnitude of the linear component of [^{3}H]dipyridamole binding was reduced up to 4-fold with a single wash (data not shown).

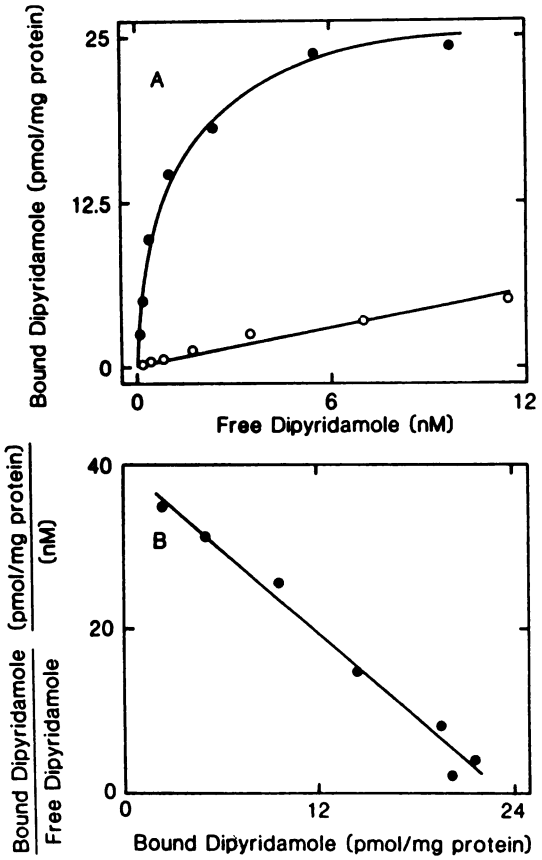

Fig. 3. Concentration dependence of [^{3}H]dipyridamole binding to human erythrocyte membranes. A. Membrane suspensions were incubated in [^{3}H]dipyridamole (0–12 nM) in the presence (○) and absence (●) of the transport inhibitor NBTGR (1 μM), as described in Materials and Methods. The amount of [^{3}H]dipyridamole bound to the membrane pellet is plotted as a function of the equilibrium free concentration of dipyridamole. B. Mass law analysis by the method of Scatchard of data for the site-specific binding of [^{3}H]dipyridamole to human erythrocyte membranes. In this instance, bound dipyridamole represents the difference between membrane-associated dipyridamole in the presence and absence of NBTGR.

The effects of varying concentrations of uridine and adenosine on saturable binding of [^{3}H]dipyridamole to human erythrocyte membranes are shown in Fig. 4. Equilibrium dipyridamole binding in the presence of adenosine and uridine was reduced in an apparent competitive manner with inhibition constants of 0.1 and 0.9 mM, respectively, values similar to that obtained previously for adenosine and uridine inhibition of NBMPR binding to human erythrocyte membranes (12, 42). Plots of apparent K_d values for dipyridamole binding in the presence of nucleoside versus initial nucleoside concentrations were linear, providing further evidence of simple competitive inhibition of binding activity. The results of further experiments presented in Fig. 5 demonstrated that the nucleoside transport inhibitors, NBMPR, NBTGR, and dilazep, effectively inhibited saturable [^{3}H]dipyridamole binding at nM concentrations, with IC_{50} values of 1.7, 2.3, and 1.7 nM, respectively. These values are probably underestimated since inhibitor depletion by the cells has not been considered.

Dipyridamole inhibition of uridine transport in the presence of NBMPR. The above data are consistent with the

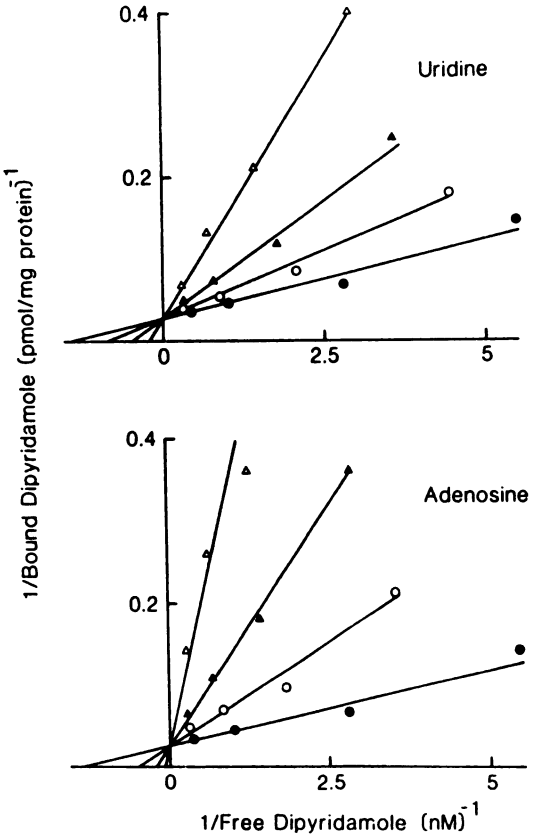

Fig. 4. Effect of adenosine and uridine on [^{3}H]dipyridamole binding to human erythrocyte membranes. The reciprocals of [^{3}H]dipyridamole bound to high affinity sites in the presence of graded concentrations of uridine and adenosine at 22° are plotted against the respective reciprocals of the free equilibrium concentrations of [^{3}H]dipyridamole. Initial concentrations of uridine (mM) were: 0 (●), 0.26 (○), 1.0 (▲), and 4.4 (△). The adenosine concentrations (mM) were: 0 (●), 0.12 (○), 0.48 (▲), and 1.9 (△). Apparent K_i values for uridine and adeonsine were 0.9 and 0.1 mM, respectively.

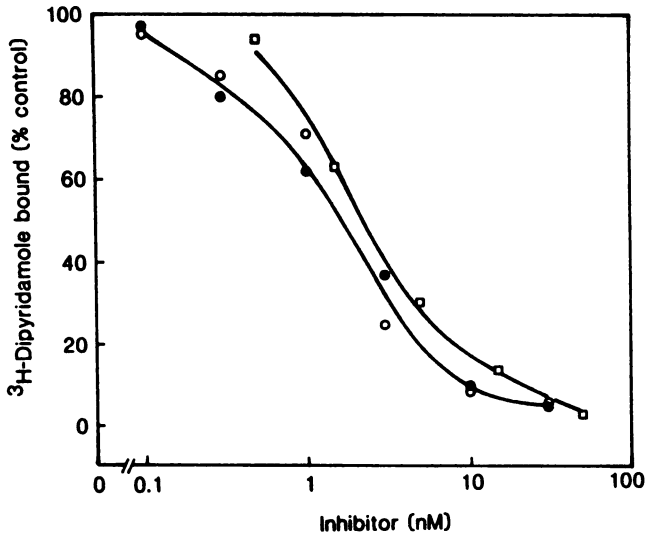

Fig. 5. Effect of nucleoside transport inhibitors on [^{3}H]dipyridamole binding to human erythrocyte membranes. High affinity binding of [^{3}H]dipyridamole (initial extracellular concentration 2 nM) to human erythrocyte membranes was determined as described in Materials and Methods. Values are the means of two separate experiments and are expressed as percentages of control [^{3}H]dipyridamole binding activities measured in the absence of inhibitor. ●, NBMPR; ○, dilazep; □, NBTGR.

TABLE 1

Inhibition of uridine entry into guinea pig erythrocytes by dipyridamole and NBMPR

The initial rates of uridine zero-*trans* entry were determined as described in Materials and Methods. The flux rates at 22° (corrected for the NBMPR-insensitive component) are compared to the predicted transport rates in the presence of two inhibitors calcualted from Eq. 1 (one inhibitory site) or Eq. 2 (two inhibitory sites). The latter calculation required that the k_m for influx be known and from earlier studies was shown to be equal to 0.066 mM at 22° (33).

Uridine Concentration	Final inhibitor concentration		Transport rates		
	Dipyridamole	NBMPR	Observed	Predicted	
				One site	Two sites
	nM		*mmol/liter of cells/hr*		
0.5 mM	0	0	0.076		
	0.48	0	0.060		
	1.72	0	0.045		
	3.90	0	0.033		
	0.48	0.25	0.034	0.035	0.018
	1.72	0.25	0.026	0.029	0.0096
	3.90	0.25	0.025	0.024	0.0057
0.12 mM	0	0	0.049		
	0.72	0	0.038		
	4.43	0	0.017		
	0.72	0.25	0.019	0.021	0.015
	4.43	0.25	0.015	0.015	0.0052

notion that NBMPR and dipyridamole either add at the same site on or near the nucleoside carrier or at separate sites linked by allosteric interaction (see Discussion). In an attempt to decide between these two mechanisms, the degree of inhibition of uridine transport produced by the inhibitors when acting alone or together was studied and the rate observed was compared with that predicted for either 1) a single inhibitory site, or 2) two noninterfering sites. The detailed equations describing these two conditions have been derived previously (43), and the important points are summarized. In the case where there is a single inhibitory site, inhibition is a function of the sum of inhibitor concentrations and of a product of inhibitor concentrations when two different inhibitor molecules can add to the carrier at the same time. Thus, the predicted value for inhibitors competing for a single site is described by the following relationship:

$$\left(\frac{V}{V_{IL}} - 1\right) = \left(\frac{V}{V_I} - 1\right) + \left(\frac{V}{V_L} - 1\right) \qquad (1)$$

where V is the rate of transport in the absence of inhibitors, V_I and V_L are the rates of transport in the presence of inhibitors I or L, respectively, and V_{IL} is the rate of transport in the presence of both I and L. In the case of two inhibitor molecules adding to the carrier at the same time, the transport rates are related by the following expression:

$$\left(\frac{V}{V_{IL}} - 1\right) = \left(\frac{V}{V_I} - 1\right) + \left(\frac{V}{V_L} - 1\right)\left(1 + \frac{[I]}{K_{LI}}\right) \qquad (2)$$

where K_{IL} is the half-saturation constant for inhibitor I in the presence of a saturating concentration of L.

The observed rates of uridine entry (extracellular uridine concentrations 0.5 and 0.12 mM) in the presence of dipyridamole, NBMPR, or both are shown in Table 1, and are compared to the predicted rates based on mechanisms involving a single inhibitory site or two separate inhibitory sites (Eqs. 1 and 2,

respectively). The data demonstrate that the predicted and calculated rates are identical if the inhibitors compete (Eq. 1), whereas if two inhibitor sites exist the observed inhibition should be much greater than it in fact is.

Discussion

Although the inhibitory effects of dipyridamole on nucleoside transport were observed more than 20 years ago (11), the mechanism by which this clinically available drug achieves this effect is largely unknown and in dispute (see the introduction). The experiments described in this paper were designed to clarify the mechanism of dipyridamole interaction with the NBMPR-sensitive erythrocyte nucleoside transporter.

Dipyridamole was found to be a potent competitive inhibitor of uridine equilibrium exchange influx into guinea pig erythrocytes. Analysis of the inhibition data using equilibrium free dipyridamole concentrations rather than total dipyridamole levels reduced the estimated apparent K_i value from 7 to 1 nM. These results are consistent with the view that dipyridamole competes directly with nucleosides for the permeation site of the transport system. In support of this conclusion is the finding of a single class of high affinity dipyridamole-binding sites on human erythrocyte membranes with an apparent K_d value of 0.65 ± 0.07 nM. Dipyridamole binding to this site is blocked by nucleosides and nucleoside transport inhibitors. Furthermore, inhibition of dipyridamole binding by uridine and adenosine was apparently competitive with inhibition constants similar to previous estimates of the apparent K_m of equilibrium exchange transport (41, 44, 45). The maximum number of high affinity dipyridamole-binding sites on human erythrocyte membranes is similar to that determined for the site-specific binding of NBMPR. Also, previous studies have shown that dipyridamole competes competitively with NBMPR for the NBMPR-binding site on human erythrocytes (18), which has also been postulated to be within the permeation site (12). These data suggest that the high affinity dipyridamole-binding site is located on or near the nucleoside transport protein, and the similarity of the apparent K_i values for nucleoside inhibition of high affinity dipyridamole binding to the apparent K_m values for nucleoside equilibrium exchange influx is further evidence that dipyridamole competes with nucleosides for the permeation site of the nucleoside transporter.

The kinetic and inhibitor binding results presented in this report would also be consistent with a more complex model of nucleoside transport in which inhibitor binding at one site induces a conformational change in the transporter, thereby allosterically altering the affinity of permeant at its site. In addition, some investigators (46) have argued that the structures of dipyridamole and NBMPR have little in common and, therefore, the binding sites for these two inhibitors must also be different. Support for this view comes from the finding that high concentrations of dipyridamole decrease the rate of NBMPR dissociation in a variety of membrane preparations (27, 42, 47). However, X-ray diffraction studies point to similarities in the three-dimensional crystal structures of NBMPR and dipyridamole,[1] and the high concentrations (>100 nM) of dipyridamole required to effect NBMPR dissociation may simply reflect nonspecific interactions with the transporter or other membrane components following partition of the lipophilic inhibitor into the membrane (42). Also, the conclusion that dipyridamole acts at a site distinct from the NBMPR-binding site, derived from the observation that 10 μM dipyridamole inhibited nucleoside transport by rat Walker 256 cells (25), whereas 1 μM NBMPR was without effect, is not applicable to the present studies on the NBMPR-sensitive nucleoside transport system. Walker 256 cells lack high affinity NBMPR-binding sites (48), and these cells exhibit pure NBMPR-insensitive nucleoside transport. Moreover, nucleoside transporters in a variety of rat tissues exhibit a low sensitivity to dipyridamole inhibition (20–22, 31, 32). Direct evidence to suggest that NBMPR and dipyridamole act at a common site comes from the studies depicted in Table 1 that clearly indicate that the transport rates observed in the presence of both NBMPR and dipyridamole are identical to those predicted for a single inhibitory site. Therefore, at the present time the available data are consistent with the simpler model that dipyridamole inhibits NBMPR-sensitive nucleoside transport in a manner similar to that of NBMPR and that both inhibitors interact at common or overlapping sites on the carrier.

The vectorial properties of dipyridamole binding to the nucleoside transport system can be determined by examining the inhibition profiles for zero-*trans* exit and entry experiments (see Ref. 49). For example, an inhibitor that interacts with the permeation site may do so at either the inner or the outward facing conformation, depending upon the location of the inhibitor and its relative affinity for the two conformations. Thus, in zero-*trans* entry experiments where the substrate is present in the suspending medium, and if an inhibitor is bound only at the external site, then inhibition of substrate entry is purely competitive. Under these circumstances inhibition of zero-*trans* exit will be noncompetitive, because the substrate in the internal pool is unable to compete with the external site. Similarly, if the inhibitor binds on both the inner and outward surfaces of the membrane, inhibition of both zero-*trans* influx and efflux will be noncompetitive. In the present case, the inhibition pattern for zero-*trans* influx is competitive but dipyridamole behaves as a noncompetitive inhibitor of zero-*trans* uridine efflux. These data suggest that the dipyridamole-binding site is largely located on the outer surface of the cell membrane. The NBMPR-binding site has also been suggested to be preferentially located on the outward-facing conformation of the substrate permeation site in guinea pig and nucleoside-permeable sheep erythrocytes (12, 17).

In conclusion, the present results suggest that dipyridamole and NBMPR interact at common or overlapping sites on the NBMPR-sensitive transporter. Furthermore, it is proposed that the inhibitor binding site for NBMPR and dipyridamole in mammalian erythrocytes is largely located on the outer surface of the cell membrane, totally or partially within the permeation site.

Acknowledgments

I thank Andrew Ng and Brad Martin for expert technical assistance.

References

1. Young, J. D., and S. M. Jarvis. Nucleoside transport in animal cells. Review. *Biosci. Rep.* **3**:309–322 (1983).
2. Paterson, A. R. P., N. Kolassa, and C. E. Cass. Transport of nucleoside drugs in animal cells. *Pharmacol. Ther.* **12**:515–536 (1981).
3. Plagemann, P. G. W., and R. M. Wohlhueter. Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzylthioinosine or hypoxanthine. *Biochim. Biophys. Acta* **773**:39–52 (1984).

[1] P. W. Codding and J. Jakana, personal communication.

4. Ritchie, J. L., and L. A. Harker. Platelet and fibrinogen coronary atherosclerosis. *Am. J. Cardiol.* **39**:595–598 (1977).
5. Needleman, P., and E. M. Johnson. Vasodilators and the treatment of angina, in *The Pharmacological Basis of Therapeutics* (A. G. Gilman, L. S. Goodman, and A. Gilman, eds.). MacMillan, New York, 819–833 (1980).
6. Zhen, Y.-S., M. S. Lui, and G. Weber. Effects of acivicin and dipyridamole on hepatoma 3924A cells. *Cancer Res.* **43**:1616–1619 (1983).
7. Kang, G.-J., and A. P. Kimball. Dipyridamole enhancement of toxicity to L1210 cells by deoxyadenosine and deoxycoformycin combinations *in vitro*. *Cancer Res.* **44**:461–466 (1984).
8. Fisher, P. H., R. Pamukcu, G. Bittner, and J. K. V. Willson. Enhancement of the sensitivity of human colon cancer cells to growth inhibition by acivicin achieved through inhibition of nucleic acid precursor salvage by dipyridamole. *Cancer Res.* **44**:3355–3359 (1984).
9. Grem, J. L., and P. H. Fischer. Augmentation of 5-fluorouracil cytotoxicity in human colon cancer cells by dipyridamole. *Cancer Res.* **45**:2967–2972 (1985).
10. Cabral, S., S. Leis, L. Bover, M. Nembrot, and J. Mordoh. Dipyridamole inhibits reversion by thymidine of methotrexate effect and increases drug uptake in Sarcoma 180 cells. *Proc. Natl. Acad. Sci. USA* **81**:3200–3203 (1984).
11. Kubler, W., and H. J. Bretschneider. Kompetitive hemmung der katalysierten adenosindiffusion als mechanismus der coronarewеiternden wirkung eines pyrimidopyrimidin derivates. *Pflueger's Arch. Ges. Physiol.* **280**:141–157 (1964).
12. Jarvis, S. M., D. McBride, and J. D. Young. Erythrocyte nucleoside transport: asymmetrical binding of nitrobenzylthioinosine to nucleoside permeation sites. *J. Physiol. (Lond.)* **324**:31–46 (1982).
13. Scholtissek, C. Studies on the uptake of nucleic acid precursors into cells in tissue culture. *Biochim. Biophys. Acta* **158**:435–447 (1968).
14. Plagemann, P. G. W. Nucleoside transport by Novikoff rat hepatoma cells growing in suspension culture. Specificity and mechanism of transport reactions and relationship to nucleoside incorporation into nucleic acids. *Biochim. Biophys. Acta* **233**:688–701 (1971).
15. Lips, J. P. M., J. J. Sixma, and A. C. Trieschnigg. Inhibition of uptake of adenosine into human blood platelets. *Biochem. Pharmacol.* **29**:43–50 (1980).
16. Turnheim, K., B. Plank, and N. Kolassa. Inhibition of adenosine uptake in human erythrocytes by adenosine-5′-carboxamides, xylosyladenine, dipyridamole, hexobendine and *p*-nitrobenzylthioguanosine. *Biochem. Pharmacol.* **27**:2191–2197 (1978).
17. Jarvis, S. M. Kinetic and molecular properties of nucleoside transporters in animal cells, in *3rd International Symposium on Adenosine*. Springer, New York, in press.
18. Hammond, J. R., A. R. P. Paterson, and A. S. Clanachan. Benzodiazepine inhibition of site-specific binding of nitrobenzylthioinosine, an inhibitor of adenosine transport. *Life Sci.* **29**:2207–2214, (1981).
19. Marangos, P. J., M. S. Finkel, A. Verma, M. F. Maturi, J. Patel, and R. E. Patterson. Adenosine uptake sites in dog heart and brain: interaction with calcium antagonists. *Life Sci.* **35**:1109–1116 (1984).
20. Wu, J.-S. R., and J. D. Young. Photoaffinity labelling of nucleoside transport proteins in plasma membranes isolated from rat and guinea-pig liver. *Biochem. J.* **220**:499–506 (1984).
21. Shi, M. M., J.-S. R. Wu, C.-M. Lee, and J. D. Young. Nucleoside transport. Photoaffinity labelling of high affinity nitrobenzylthioinosine binding sites in rat and guinea pig lung. *Biochem. Biophys. Res. Commun.* **118**:594–600 (1984).
22. Williams, E. F., P. H. Barker, and A. S. Clanachan. Nucleoside transport in heart: species differences in nitrobenzylthioinosine binding, adenosine accumulation, and drug induced potentiation of adenosine action. *Can. J. Physiol. Pharmacol.* **62**:31–37 (1984).
23. Paterson, A. R. P., E. Y. Lau, E. Dahlig, and C. E. Cass. A common basis for inhibition of nucleoside transport by dipyridamole and nitrobenzylthioinosine. *Mol. Pharmacol.* **18**:40–44 (1980).
24. Wohlhueter, R. M., W. E. Brown, and P. G. W. Plagemann. Kinetic and thermodynamic studies on nitrobenzylthioinosine binding to the nucleoside transporter of Chinese hamster ovary cells. *Biochem. Biophys. Acta* **731**:168–176 (1983).
25. Belt, J. A., and L. D. Noel. Nucleoside transport in Walker 256 rat carcinosarcoma and S49 mouse lymphoma cells. Differences in sensitivity to nitrobenzylthioinosine and thiol reagents. *Biochem. J.* **232**:681–688 (1985).
26. Plagemann, P. G. W., and R. M. Wohlhueter. Nitrobenzylthioinosine-sensitive and resistant nucleoside transport in normal and transformed rat cells. *Biochim. Biophys. Acta* **816**:381–395 (1985).
27. Koren, R., C. E. Cass, and A. R. P. Paterson. The kinetics of dissociation of the inhibitor of nucleoside transport, nitrobenzylthioinosine, from high-affinity binding sites of cultured hamster cells. *Biochem. J.* **216**:299–308 (1983).
28. Gerlach, E., B. Deuticke, and J. Duhm. Inhibition of phosphate uptake by dipyridamole-efflux greater reduced than influx. Adenosine, inosine, guanosine have no effect on influx but reduce efflux. *Pflueger's Arch. Ges. Physiol.* **280**:243–274 (1964).
29. Plagemann, P. G. W., and D. P. Richey. Transport of nucleosides, nucleic acid bases, choline and glucose by animal cells in culture. *Biochim. Biophys. Acta* **344**:263–305 (1974).
30. Davies, L. P., and V. Huston. Peripheral benzodiazepine binding sites in heart and their interaction with dipyridamole. *Eur. J. Pharmacol.* **73**:209–211 (1981).
31. Kolassa, N., and K. Pfleger. Adenosine uptake by erythrocytes of man, rat and guinea-pig and its inhibition by hexobendine and dipyridamole. *Biochem. Pharmacol.* **24**:154–156 (1975).
32. Jarvis, S. M., and J. D. Young. Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and *p*-chloromercuriphenylsulphonate. *J. Membr. Biol.*, in press.
33. Jarvis, S. M., and B. W. Martin. Effects of temperature on the transport of nucleosides in guinea pig erythrocytes. *Can. J. Physiol. Pharmacol.* **64**:193–198 (1986).
34. Ross, H., and K. Pfleger. Kinetics of adenosine uptake by erythrocytes, and the influence of dipyridamole. *Mol. Pharmacol.* **8**:417–425 (1972).
35. Jarvis, S. M. Inhibition of nucleoside transport by dipyridamole. *Can. J. Physiol. Pharmacol.* **64**:AXIV (1986).
36. Jarvis, S. M., and J. D. Young. Nucleoside transport in human erythrocytes: evidence that nitrobenzylthioinosine binds specifically to functional nucleoside transport sites. *Biochem. J.* **190**:373–376 (1980).
37. Dixon, M. The determination of enzyme inhibitor constants. *Biochem. J.* **55**:160–171 (1953).
38. Cornish-Bowden, A. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem. J.* **137**:143-144 (1974).
39. Cass, C. E., L. A. Gaudette, and A. R. P. Paterson. Mediated transport of nucleosides in human erythrocyte membrane. *Biochim. Biophys. Acta* **345**:1–10 (1974).
40. Jarvis, S. M., J. R. Hammond, A. R. P. Paterson, and A. S. Clanachan. Species differences in nucleoside transport: a study of uridine transport and nitrobenzylthioinosine binding by mammalian erythrocytes. *Biochem. J.* **208**:83–88 (1982).
41. Plagemann, P. G. W., R. M. Wohlhueter, and J. Erbe. Nucleoside transport in human erythrocytes. A simple carrier with directional symmetry and differential mobility of loaded and empty carrier. *J. Biol. Chem.* **257**:12069–12074 (1982).
42. Jarvis, S. M., S. N. Janmohamed, and J. D. Young. Kinetics of nitrobenzylthioinosine binding to the human erythrocyte nucleoside transporter. *Biochem. J.* **216**:661–667 (1983).
43. Deves, R., and R. M. Krupka. Testing transport systems for competition between pairs of reversible inhibitors. Inhibition of erythrocyte glucose transport by cytochalasm B and steroids. *J. Biol. Chem.* **255**:11870–11874 (1980).
44. Jarvis, S. M., J. R. Hammond, A. R. P. Paterson, and A. S. Clanachan. Nucleoside transport in human erythrocytes. A simple carrier with directional symmetry in fresh cells but with directional asymmetry in cells from outdated blood. *Biochem. J.* **210**:457–461 (1983).
45. Plagemann, P. G. W., R. M. Wohlhueter, and M. Kraupp. Adenosine uptake, transport and metabolism in human erythrocytes. *J. Cell. Physiol.* **125**:330–336 (1985).
46. Paterson, A. R. P., E. S. Jakobs, E. R. Harley, N. W. Fu, M. J. Robbins, and C. E. Cass. Inhibition of nucleoside transport, in *Regulatory Functions of Adenosine* (R. M. Berne, T. W. Rall, and R. Rubio, eds.). Martinus Nijhoff, The Hague, 203–220 (1983).
47. Hammond, J. R., and A. S. Clanachan. Species differences in the binding of ^{3}H-nitrobenzylthioinosine to the nucleoside transport system in mammalian central nervous system membranes: evidence for interconvertible conformations of the binding site/transporter complex. *J. Neurochem.* **45**:527–535 (1985).
48. Paterson, A. R. P., E. S. Jakobs, E. R. Harley, C. E. Cass, and M. J. Robins. Inhibitors of nucleoside transport as probes and drugs, in *Development of Target-Oriented Cancer Drugs*, (Y.-C. Cheng, B. Goz, and M. Minkoff, eds.). Raven Press, New York, 41–56 (1983).
49. Deves, R., and R. M. Krupka. A new approach in the kinetics of biological transport. The potential of reversible inhibition studies. *Biochim. Biophys. Acta* **510**:186–200 (1978).

Send reprint requests to: Dr. Simon M. Jarvis, Biological Laboratory, University of Kent, Cantebury, Kent CT2 7NJ, U.K.