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A Common Basis for Inhibition of Nucleoside Transport by Dipyridamole and Nitrobenzylthioinosine?

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SUMMARY

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Transport of uridine by monolayer cultures of HeLa cells was inhibited by nitrobenzylthioinosine, dipyridamole, and lidoflazine. Biphasic concentration–effect curves were obtained for inhibition of nucleoside transport by nitrobenzylthioinosine, but not for inhibition by dipyridamole. Dipyridamole and lidoflazine interfered with high-affinity binding of [^{3}H]nitrobenzylthioinosine to HeLa cells in an apparently competitive fashion; values of 1, 30, and 300 nM were obtained for dissociation constants, respectively, for nitrobenzylthioinosine, dipyridamole, and lidoflazine. The apparent competition with nitrobenzylthioinosine at the latter's high-affinity binding sites suggests that dipyridamole and lidoflazine inhibit nucleoside transport by interaction with these sites.

INTRODUCTION

Contributing to the uptake of uridine by cultured cells are (a) mediated entry via the NBMPR[1]-sensitive, nucleoside-specific transport mechanism(s), and (b) an uncharacterized, NBMPR-insensitive component of uptake which might include diffusion or high-K_m mediated processes (1). Recent studies of uridine permeation which employed rapid sampling procedures have demonstrated the entry of uridine by way of saturable transport processes in various cell types; K_m values of about 50–500 μM have been reported (2–4). Facilitated diffusion characteristics have been shown for this process under conditions in which uridine phosphorylation was impaired (2), and NBMPR inhibition of uridine transport has been shown in various cell types (1, 5–7). The entry of uridine into HeLa monolayer cells by a low-K_m (2–4 μM), NBMPR-sensitive transport process has also been shown by an initial rate method (1).

NBMPR and related compounds are potent inhibitors of nucleoside transport in various types of animal cells (1, 5–10). NBMPR is bound reversibly, but with high affinity, to specific sites on HeLa cells, presumably on the plasma membrane (11). NBMPR saturation of these sites, approached in medium containing 5 nM NBMPR, resulted in only partial inhibition of nucleoside transport, whereas at 5 μM NBMPR, the saturable, low-K_m uridine transport process was inhibited and a remaining, NBMPR-insensitive process was then apparent (1, 11).

A variety of compounds of diverse structures unrelated to nucleosides have been recognized as inhibitory to nucleoside uptake, apparently through inhibition of transport; these are discussed by Berlin and Oliver (10). In the present study, two such inhibitors of adenosine permeation, dipyridamole (12–14) and lidoflazine (15), are compared with NBMPR and are shown to compete with NBMPR for binding to the high-affinity sites of HeLa cells.

MATERIALS AND METHODS

The uptake of [5-^{3}H]uridine by HeLa cell monolayers during intervals of 10 s was measured by a previously described procedure employing replicate monolayer cultures (1), each of which contained 10^6 exponentially proliferating cells in a 2-oz prescription bottle. Rates of uridine uptake determined in this way were considered to be initial rates (and, therefore, uridine transport rates) because under the conditions here employed, time courses of uridine uptake by the monolayers extrapolated through zero time and rates of uptake were constant for several minutes (1).

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[1] Abbreviations used: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBTGR, 2-amino-6-[(4-nitrobenzyl)thiol]-9-β-D-ribofuranosylpurine; dipyridamole (Persantine), 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine; lidoflazine, 4-[4,4-bis(4-fluorophenyl)butyl]-*N*-(2,6-dimethylphenyl)-1-piperazinacetamide; MEM-T, Eagle's minimal essential medium without $NaHCO_3$, but supplemented with 12 mM NaCl and 20 mM *N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer, pH 7.4.

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Uptake intervals were ended by flooding the cell sheets with cold 0.15 M NaCl. Cultures were processed individually through this procedure and all assays were performed in triplicate. After removal of the cold saline stopping solution and thorough drainage, cell sheets were dissolved in 1.5-ml portions of 0.5 M KOH, and after the addition of 15 ml of a xylene–detergent fluor (16), the resulting solutions were assayed for ^3H by liquid scintillation counting. The 10-s uridine uptake rates were corrected for uridine uptake during intervals of zero seconds, as described previously (1).

In determining the influence of NBMPR, dipyridamole, and lidoflazine on uridine uptake rates, monolayer cultures were exposed at 20°C to 6.0-ml portions of graded concentrations of these inhibitors in MEM-T medium (a modified, serum-free culture medium (1)) for 20-min intervals (see below). At the end of the 20-min interval, three 4.0-ml portions of the inhibitor-containing medium from the cultures of each triplicate set were pooled and a 10.0-ml portion of the pool was added to a lyophilized residue of [5-^3H]uridine, to make a solution for measurement of the cellular rate of uridine uptake in the presence of equilibrium concentrations of free and bound inhibitor. The 10-s assay of [5-^3H]uridine uptake was performed immediately thereafter using each uridine–inhibitor solution on the same set of cultures from which the inhibitor-containing medium was taken. Cultures were processed individually through these procedures to assure precision in uptake and washing intervals.

The influence of dipyridamole and lidoflazine on binding of NBMPR to the monolayer cells was assayed as follows. Replicate monolayer cultures were incubated at 20°C for 20 min with MEM-T medium containing graded concentrations of [G-^3H]NBMPR with and without specified concentrations of dipyridamole or lidoflazine. Preliminary experiments established that at 20°C an interval of 20 min was required for cell-bound [G-^3H]NBMPR to reach equilibrium with the medium content of [G-^3H]-NBMPR at the lowest NBMPR concentrations employed (about 0.5 nM). At the end of the assay interval, medium samples were reserved for assay of [G-^3H]-NBMPR content using liquid scintillation counting under the conditions already described, and after removal of the remaining medium by suction, the culture bottles were rinsed with 60 ml of cold 0.15 M NaCl and then drained. The monolayers were then assayed for ^3H content by the liquid scintillation procedure described above.

RPMI 6410 cells (17) were grown in static culture in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). The cultures (25 ml) in loosely capped 50-ml bottles were incubated at 37°C in humidified 5% CO_2–air. Cell concentrations were kept below 5×10^5 cells/ml by dilution with fresh medium.

Cell culture materials were obtained from GIBCO Canada Ltd., Burlington, Ontario, and ^3H-labeled nucleosides from Moravek Biochemicals (City of Industry, Calif.). Dipyridamole was provided by Boehringer-Ingleheim (Canada) Ltd., Dorval, Quebec, and lidoflazine was a gift from Dr. H. Van Belle, Jannsen Pharmaceutica, Beerse, Belgium. Arabinosyladenine, arabinosylcytosine, nebularine, and 3-deazauridine were provided by the Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). Tubercidin was purchased from Sigma Chemical Company (St. Louis, Mo.). NBMPR and NBTGR were prepared in this laboratory (18) from 6-thioinosine and 6-thioguanosine provided by the Division of Cancer Treatment, National Cancer Institute.

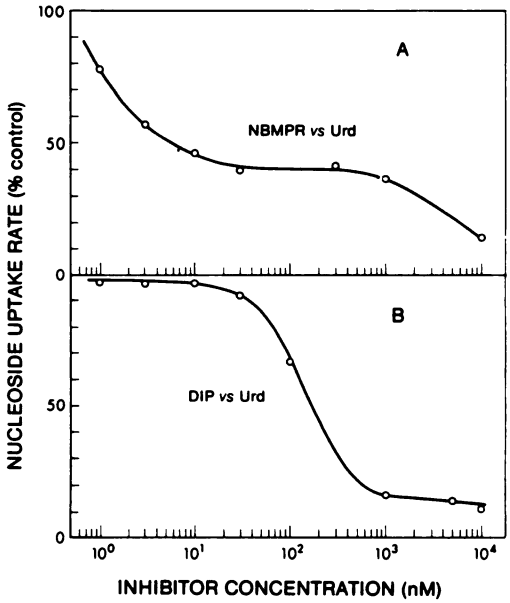

FIG. 1. *Inhibition of nucleoside uptake by NBMPR and dipyridamole*

Replicate monolayer cultures of HeLa cells were incubated for 20 min in MEM-T medium containing NBMPR or dipyridamole (DIP) at the indicated concentrations. A 10.0-ml pool of postincubation medium was obtained from each triplicate set of cultures and to each such pool [5-^3H]uridine was added to achieve a final concentration of 4 μM. Each of the resulting solutions of [5-^3H]uridine was used to measure uridine uptake during 10-s intervals by the monolayers of the triplicate set of cultures from which that inhibitor-containing medium was obtained. The uptake of [5-^3H]uridine was measured as described under Materials and Methods and was not corrected for nonspecific uptake of uridine, as in a preceding report (1). Rates of uridine uptake (pmol/10^6 cells/10 s) in the absence of inhibitors (100%) were: (A) 2.8 and (B) 3.1.

RESULTS AND DISCUSSION

It is evident in Fig. 1 that dipyridamole is a potent inhibitor of uridine transport by HeLa monolayer cells; the uridine uptake rate was reduced to 50% of the control value in the presence of 200 nM dipyridamole. The sigmoidal curve (Fig. 1B), which describes the relationship between dipyridamole concentration (log scale) and inhibition of uridine uptake, is simpler than the biphasic concentration–effect relationship obtained when NBMPR was the inhibitor of uridine uptake (Fig. 1A). The biphasic concentration–effect relationship between NBMPR concentration and the inhibition of uridine transport has been demonstrated repeatedly in experiments like that of Fig. 1 with HeLa monolayers. A result similar to that of Fig. 1A was also obtained when the transport of uridine by HeLa cells in suspension was measured by a time-course method.[2]

[2] Employed was a rapid sampling procedure (9) in which intervals of [5-^3H]uridine uptake by HeLa cells in suspension were terminated by

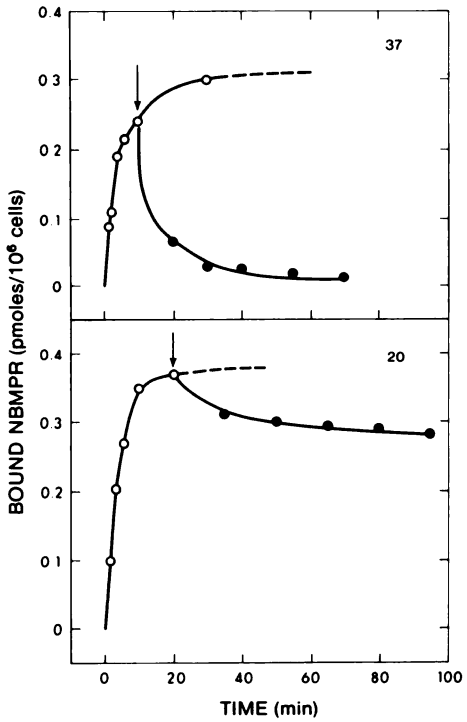

FIG. 2. *Displacement of NBMPR from HeLa cells by dipyridamole*

Replicate monolayer cultures were incubated with 4.0-ml portions of MEM-T medium containing [G-^{3}H]NBMPR (1 nM) for the indicated intervals, at which time monolayers were washed with cold 0.15 M NaCl and the cell content of [G-^{3}H]NBMPR was determined as described in Materials and Methods. At the times indicated by arrows, particular cultures (●) were made 25 μM in dipyridamole by the addition of 0.1 ml of 1 mM dipyridamole in MEM-T and incubations were continued for the intervals shown. Plotted on the ordinate is the cell content of [G-^{3}H]NBMPR in the absence (○) and presence (●) of 25 μM dipyridamole.

That portion of the Fig. 1A concentration–effect curve concerned with NBMPR concentrations lower than 10 nM describes the effects of NBMPR occupancy of the high-affinity NBMPR binding sites (11). Increases in NBMPR concentrations of about 1000-fold were required to inhibit the component of uridine transport that remained functional after NBMPR saturation of the high-affinity sites. The nature of the low-affinity interaction between NBMPR and the nucleoside transport mechanism(s) is not understood. Biphasic concentration–effect curves for NBMPR inhibition of nucleoside uptake were not seen when NBMPR and nucleoside permeants were presented to cells simultaneously (1, 19), apparently because the approach to equilibrium in NBMPR binding at low NBMPR concentrations required intervals longer than those employed in measurement of nucleoside uptake rates.

adding NBMPR (final concentration, 5 μM); immediately after addition of NBMPR, cells were pelleted centrifugally under an oil layer. Time courses of uridine uptake (2–10 s) measured by this method extrapolated through time-zero uptake values (NBMPR "stopper" added prior to or simultaneously with [5-^{3}H]uridine) and initial rates were determined graphically. Initial rates of uridine uptake so measured in this system are considered to be uridine transport rates (Dahlig, E., Lau, E. Y., Cass, C. E., and Paterson, A. R. P. to be published).

To test the possibility that the inhibitory effects of dipyridamole and NBMPR on uridine transport might result from the interaction of either agent with the same sites on the nucleoside transport mechanism, we examined the influence of dipyridamole on NBMPR binding by replicate HeLa cell monolayers. The experiment of Fig. 2 demonstrated displacement of NBMPR from specific binding sites on HeLa cells by dipyridamole, which was added (final concentration, 25 μM) at the times indicated by the arrows. The displacement of NBMPR by dipyridamole was more rapid at 37°C than at 20°C; the higher capacity of HeLa cells for NBMPR binding at 20°C relative to that at 37°C has been reported previously. Also apparent in Fig. 2 is the time dependence of the approach to equilibrium between cell-bound [G-^{3}H]NBMPR and [G-^{3}H]NBMPR in the medium.

The experiment of Fig. 3 demonstrated that the binding of NBMPR to HeLa cells was reduced in the presence of dipyridamole and suggested that dipyridamole competed with NBMPR at the latter's high-affinity binding site. Under the conditions of this experiment (1.0 nM NBMPR, 20°C), dipyridamole at about 0.1 μM reduced the cellular content of specifically-bound NBMPR to half of the control value.

The experiment of Fig. 4 assessed the influence of NBMPR concentration on the site-specific binding of NBMPR by HeLa cells. Mass law analysis of this binding data by the reciprocal plot method (20) yielded linear plots (Fig. 4) in the presence or absence of dipyridamole. The NBMPR dissociation constant (K_{dA}) in this experiment was 1 nM. It is apparent in Fig. 4 that NBMPR binding was reduced in the presence of dipyridamole in a manner that was related to the latter's concentration; the apparent convergence at the ordinate of the reciprocal plots obtained at several dipyridamole concentrations

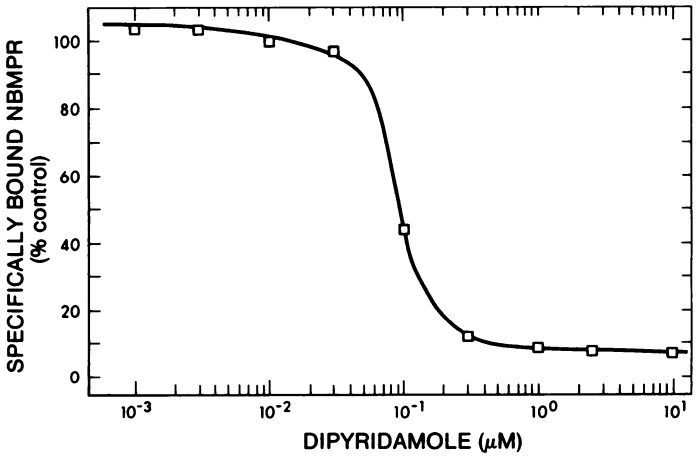

FIG. 3. *Inhibition by dipyridamole of NBMPR binding by HeLa cells*

Replicate monolayer cultures were incubated at 20°C for 20 min in MEM-T medium containing 1 nM [G-^{3}H]NBMPR and the indicated concentrations of dipyridamole; the cellular content of [G-^{3}H]NBMPR, determined as described under Materials and Methods, was corrected for nonspecific retention of [G-^{3}H]NBMPR by subtraction of the cellular uptake of the latter in the presence of 5 μM NBTGR (11); this correction represented 0.003 pmol NBMPR/10^6 cells. In the absence of dipyridamole, the cell content of specifically bound NBMPR was 0.31 pmol/10^6 cells.

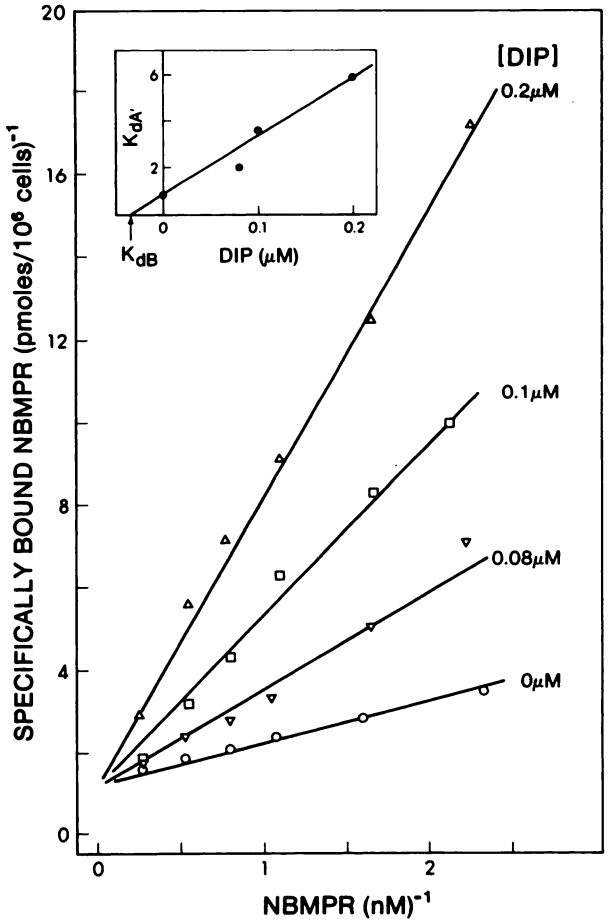

FIG. 4. *Competitive inhibition by dipyridamole of NBMPR binding to HeLa cells*

Replicate monolayer cultures were incubated at 20°C for 20 min with graded concentrations of [G-^{3}H]NBTGR in MEM-T with no other additives (control, ○) or with 5 μM NBMPR (not shown), 0.08 μM dipyridamole (DIP) (▽), 0.1 μM dipyridamole (□), or 0.2 μM dipyridamole (△). At the end of incubation intervals, medium samples were taken for determination of [G-^{3}H]NBMPR content and monolayers were washed with cold 0.15 M NaCl prior to determination of cellular [G-^{3}H]NBMPR content as described under Materials and Methods. The latter values were corrected by subtraction of the cellular content of [G-^{3}H]NBMPR acquired in the presence of 5 μM NBTGR, a measure of nonspecific cellular retention of NBMPR (11). In the figure, reciprocals of the cellular content of specifically-bound NBMPR are plotted against the concentration of free NBMPR in the medium. Straight lines were fitted to these data and from the abscissa intercepts (i) for data from control cultures, the NBMPR dissociation constant (K_{dA}) was determined, and (ii) for data from dipyridamole-treated cultures, the apparent dissociation constants ($K_{dA'}$) were obtained. From Edsall and Wyman's application of mass law analysis to binding data (20), the following relationship may be derived in which K_{dB} is the dissociation constant of a ligand B (dipyridamole) which competes with ligand A (NBMPR) for binding at a common site, and [B] is the concentration of ligand B (dipyridamole): $K_{dA'} = K_{dA} + (K_{dA}/K_{dB})\cdot[B]$. This relationship indicates that $K_{dA'}$ is a linear function of [B].

suggested that dipyridamole competed with NBMPR at the latter's binding site. In the Fig. 4 inset, apparent NBMPR dissociation constants ($K_{dA'}$), measured in the presence of particular dipyridamole concentrations, are plotted against the latter. The linearity of this replot indicates that dipyridamole competed with NBMPR at

TABLE 1

Protection of RPMI 6410 cells by dipyridamole against growth inhibition by toxic nucleosides

RPMI 6410 cells were cultured in medium without additives (controls) or in the same medium containing a specified concentration of a toxic nucleoside with and without 10 μM dipyridamole. Cell concentrations were determined daily. Proliferation rates, determined as the number of population doublings in 72 h of culture, are listed as percentages of control rates; the latter ranged between 3.6 and 4.0 doublings in 72 h.

Toxic nucleoside		Proliferation rate (% control) in the absence or presence of 10 μM dipyridamole	
Name	Concn (μM)	Absence	Presence
None			90.6
Arabinosyladenine	100	3.4[a]	81.8
Arabinosylcytosine	3	−8.9[b]	81.8
3-Deazauridine	10	8.0	100
Nebularine	1	22.1	95.1
Tubercidin	0.3	−26.5	90.4

[a] In the presence of 5 μM NBMPR (in place of dipyridamole as the protecting agent), cell proliferation took place at 75% of the control rate.

[b] Negative values indicate that cell numbers declined during the interval of culture.

the binding site (21), and from the abscissa intercept, the dipyridamole dissociation constant at that binding site was determined to be 30 nM, a value about 30-fold larger than that for NBMPR. In a related experiment, HeLa monolayers were pretreated for 20 min at 20°C with dipyridamole at graded concentrations in MEM-T medium; the monolayers were then rinsed briefly with 6 ml of MEM-T medium and assayed for NBMPR binding in the absence of dipyridamole. Analysis of the binding data by the reciprocal plot method (as in Fig. 4) gave essentially the same results as the experiment of Fig. 4, including a similar value for the dissociation constant of dipyridamole at the NBMPR binding site.[3] Analysis of these data by Hanes-Woolf and Eisenthal-Cornish Bowden plots (21) also showed apparent competition between dipyridamole and NBMPR binding.

Inhibition by the adenosine transport inhibitor, lidoflazine,[4] of NBMPR binding in the HeLa cell monolayer system was demonstrated (data not shown) in experiments similar to those with dipyridamole (Fig. 3); it was apparent that the extent of inhibition was related to lidoflazine concentration. In an experiment similar to that of Fig. 4, mass law analysis of data for the lidoflazine inhibition of NBMPR binding indicated that, as with dipyridamole, lidoflazine competed with NBMPR for binding at the latter's high-affinity sites and the apparent dissociation constant for lidoflazine at these binding sites was about 300 nM.

The experiments summarized in Table 1 illustrate for

[3] Dissociation constants for NBMPR and dipyridamole at the NBMPR binding site (see K_{dA} and K_{dB} Fig. 4) obtained in this experiment were 0.5 and 27 nM, respectively.

[4] Adenosine transport (at concentrations from 0.3 to 25 μM) by monolayer HeLa cells was inhibited by lidoflazine in experiments conducted under conditions similar to those of Fig. 1 (Paterson, A. R. P., Lau, E. Y., Dahlig, E., and Cass, C. E. unpublished results).

dipyridamole another aspect of the inhibition of nucleoside permeation, the previously described "protection" of cultured cells against cytotoxic nucleosides (8, 9). We have reported that in culture medium containing 5 μM NBMPR, animal cells proliferated, in some instances at control rates, in the presence of otherwise cytotoxic concentrations of nucleoside drugs (8, 9); the NBMPR protection effect was attributed to NBMPR blockade of the nucleoside transport mechanism which evidently mediated entry of the toxic agents into the cultured cells. It is seen that 10 μM dipyridamole protected RPMI 6410 cells against inhibitory concentrations of several nucleoside drugs during 72-h intervals of culture. The analogy between dipyridamole and NBMPR interactions with the nucleoside transport mechanisms is strengthened by these results.

The apparent competition between dipyridamole and NBMPR at the latter's high-affinity binding sites suggests that both agents bind at the same site and that inhibition of nucleoside transport by dipyridamole, at least in part, results from interaction with these sites. The NBMPR concentration–effect relationship with respect to uridine transport is biphasic, as seen in Fig. 1; the upper limb of this curve is attributable to NBMPR binding at the high-affinity sites (at which NBMPR and dipyridamole evidently compete). NBMPR inhibition of the component of nucleoside transport activity remaining after NBMPR occupation of the high-affinity sites conceivably might involve NBMPR interaction at different, low-affinity sites. The observation that the sigmoidal curve which illustrates the dipyridamole concentration–effect relationship with respect to uridine transport (Fig. 1B) is different from the biphasic NBMPR curve would seem to argue against identity of the NBMPR and dipyridamole binding sites. We have no explanation for this apparent anomaly, but suggest that occupancy of a site on the transport mechanism by either NBMPR or dipyridamole might not have the same ultimate consequences with respect to transport activity because of differences in the chemical structures of the two inhibitors.

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