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COMPETITION OF NUCLEOSIDE TRANSPORT INHIBITORS WITH BINDING OF 6-[(4-NITROBENZYL)-MERCAPTO]PURINE RIBONUCLEOSIDE TO INTACT ERYTHROCYTES AND GHOST MEMBRANES FROM DIFFERENT SPECIES

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Abstract—The potency of nucleoside transport inhibitors, including 6-[(4-nitrobenzyl)-mercapto]purine ribonucleoside (NBMPR), dilazep, mioflazine and its derivatives soluflazine and R57974 as inhibitors of the binding of [^{3}H(G)]NBMPR to intact erythrocytes and respective ghost membranes from human, mouse and hamster was determined. There was no close agreement between the IC_{50} profiles for the different inhibitors when comparing values obtained for intact cells and membranes from each species, and there was no consistent profile of differences when considering individual drugs and comparing their actions in the three species. Present data also were compared with potency values obtained previously with the same drugs directly in nucleoside transport inhibition studies with erythrocytes from the same species as well as with [^{3}H(G)]NBMPR binding studies in isolated liver and lung membranes from hamster. The overall conclusion from this and previous studies is that the evaluation of relative potencies in screening of potential nucleoside transport inhibitors is best carried out at the level of actual nucleoside transport studies in intact cells, since [^{3}H(G)]NBMPR binding studies yield discrepant data.

The facilitated, carrier-mediated transport of nucleosides across mammalian cell membranes can be inhibited by a number of drugs including nucleoside derivatives such as (6-[(4-nitrobenzyl)-mercapto]-purine ribonucleoside (NBMPR†) and non-nucleoside compounds including dipyridamole and dilazep [1–3]. NBMPR has been shown to protect mice against the deleterious effects of cytotoxic nucleoside derivatives or analogs [4, 5]. This host protecting action has been exploited in the experimental chemotherapy of certain malignancies [6, 7] and parasitic infections [8–11] in mice, using combinations of cytotoxic nucleoside and transport inhibitor (NBMPR). The rationale of this therapeutic approach is based on the findings that parasites and certain tumor cells possess nucleoside transport systems not inhibitable by NBMPR, so that this drug combination specifically restricted the cytotoxic action of the nucleoside to these latter organisms or cells.

We have been interested in further exploring the use of the above combination therapy principle against parasitic infections, and initially have focussed on the selection of pharmacokinetically most promising nucleoside transport inhibitors, considering that effective host protection against the action of cytotoxic nucleosides is clinically of vital importance. The relative potency of potential nucleoside transport inhibitors thus was assessed in adenosine transport studies using isolated erythrocytes from different species [12] as well as by competition binding studies with isolated membranes from different tissues using [^{3}H(G)]NBMPR as a ligand [13] presuming the latter to interact specifically with functional nucleoside transporter sites in membranes (c.f. Ref. 14). Some of the relative potency data obtained by the two procedures suggested that while the orders of potencies were identical or similar, the magnitude of absolute potencies and relative potency differences were not consistent with the view that inhibitor effects on adenosine transport in intact cells and on [^{3}H(G)]NBMPR binding to isolated membranes identified the same binding site(s). This prompted us to systematically explore the question of whether competition with [^{3}H(G)]NBMPR binding in intact cells and isolated membranes from one and the same cell system yielded concordant or discrepant potency data.

Thus we chose erythrocytes and erythrocyte ghosts for such a comparative study. In human erythrocytes it has been documented that a nucleoside transporter system with high sensitivity to the inhibitor NBMPR is present and that the NBMPR high affinity sites are associated with the nucleoside transport system [15, 16]. In addition to human erythrocytes, we also studied the binding of [^{3}H(G)]NBMPR to intact cells and ghost membranes from mouse and hamster erythrocytes in the presence and absence of transport inhibitors since these species are experimental models for parasitic infections susceptible to combination treatment, i.e. *Schistosoma mansoni* (mouse) and *Schistosoma haematobium* (hamster) (c.f. Ref. 17). The nucleoside transport inhibitors included in this study were a number of mioflazine derivatives whose properties were compared to those of NBMPR and dilazep.

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† Abbreviations: NBMPR (6-[(4-nitrobenzyl)-mercapto]purine ribonucleoside); HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Materials

Mioflazine (3-(aminocarbonyl)-4-[4,4-bis-(4-fluorophenyl)butyl] - *N* - (2,6 - dichlorophenyl) - 1 - piperazineacetamide·2HCl); soluflazine (3-(aminocarbonyl) - 4 - (2,6 - dichlorophenyl) - 4 - [4 - fluoro - phenyl) - 4 - (3 - pyridinylbutyl] - *N* - (2,6 - dichlorophenyl) - 1 - piperazineacetamide·2HCl) and R57974 (2-aminocarbonyl) - 4 - [5,5 - bis(4 - fluorophenyl)phen - ethyl] - *N* - (2,6 - dichlorophenyl) - 1 - piperazineacetamide·2HCl) were gifts from Dr H. Van Belle, Janssen Pharmaceutica (B-2340 Beerse, Belgium). Dilazep (*N*,*N*'-bis-[3-(3,4,5-trimethoxybenzoyloxy)-propyl]-homopiperazine was kindly provided by F. Hoffmann–La Roche Co. (Basel, Switzerland). Non-radioactive NBMPR was a gift from Prof. A. R. P. Paterson, The University of Alberta, Cancer Research Unit, Edmonton, Canada. [^{3}H(G)]NBMPR (G = generally labeled) with a specific activity of 23 Ci/mmol and radiochemical purity above 98% was purchased from Moravek Biochemicals (Brea, CA). NBMPR, mioflazine and its derivatives were dissolved in dimethyl sulphoxide at a concentration of 10 mM and initially diluted into Tris–HCl buffer (10 mM, pH 7.4) to a concentration of 10 μM.

Blood collection

Blood samples were collected by cardiac puncture (mouse and hamster) or by venous puncture (human) into heparinized tubes. Erythrocytes were washed three times with phosphate-buffered saline and the buffy coat discarded. The erythrocytes were resuspended in phosphate-buffered saline at a concentration of 2–5 $\times$ 10^8 cells per mL.

Erythrocyte ghost preparation

Erythrocytes were concentrated in phosphate-buffered saline/glucose (130 mM NaCl, 2.7 mM KCl, 7.4 mM phosphate, 113 mM D-glucose, pH 7.4) and osmotically shocked by adding a 40-fold volume of 5 mM sodium phosphate buffer for 5 min at 22° (pH 8.5) [18]. The suspension was centrifuged at 10,000 *g* for 30 min and the resulting pellet washed five times with the phosphate buffer. The final colorless pellet contained unsealed erythrocyte ghosts.

NBMPR binding assay

Whole cells. The procedure used by Clanachan and Serignese [19] was used with modifications. Erythrocyte suspensions (100 μL containing approximately 2×10^7 cells) were incubated with [^{3}H(G)]NBMPR in 50 mM Tris–HCl buffer pH 7.4 in a total volume of 400 μL at room temperature (22°). Nonspecific binding was determined in the presence of 10 μM unlabeled NBMPR. The incubations were for 30 min and the binding reactions were terminated by centrifugation through a layer of 400 μL dibutyl phthalate at 14,000 *g* for 1 min in an Eppendorf 5414 microcentrifuge. The erythrocytes were washed once with ice-cold buffer and lysed overnight with 100 μL 1% Triton X-100. After the addition of 100 μL 5% trichloroacetic acid, the precipitates were pelleted by centrifugation and radioactivity in the supernatants was determined by scintillation counting using Optifluor scintillant (Amersham Corp., Arlington Heights, IL) with external standard quench correction.

Erythrocyte ghosts. Ghost membranes (0.06–0.1 mg) were incubated with [^{3}H(G)]NBMPR in a total volume of 400 μL containing 5 mM sodium phosphate and 0.9% NaCl (pH 7.4), with and without 5 μM unlabeled NBMPR to determine nonspecific binding. Incubations were carried out at 22° for 30 min and were terminated by addition of 1 mL ice-cold saline solution rapidly followed by centrifugation at 14,000 *g* for 1 min in an Eppendorf 5414 microcentrifuge. The pelleted membranes were rinsed twice with 2 mL ice-cold saline solution, without disturbing the pellets, and then dissolved in 0.1 mL 3 N KOH overnight. Radioactivity was counted as described above.

In studies of the concentration dependence of binding, a range of 0.25 to 7.5 nM [^{3}H(G)]NBMPR was used. In competition binding studies with both intact erythrocytes and ghost membranes, 1 nM [^{3}H(G)]NBMPR was used and the concentration range for competing ligands (cold NBMPR, dilazep, mioflazine, soluflazine and R57974) was between 1 nM and 100 μM. The concentration of competing drugs causing 50% inhibition (IC_{50}) of [^{3}H(G)]NBMPR binding to cells or ghost membranes was interpolated from semi-log plots of drug concentration versus inhibition of specific binding. K_d and B_{max} values for specific binding of [^{3}H(G)]NBMPR binding to cells and membranes were derived from Scatchard plots (using software from Biosoft-Elsevier—"Dose effect analysis by microcomputers"), and in the case of studies with isolated erythrocytes B_{max} values were reported as the number of binding sites per cell, based on the number of erythrocytes and the specific radioactivity of the ligand used.

RESULTS AND DISCUSSION

Binding parameters for intact erythrocytes and ghost membranes

The binding parameters of [^{3}H(G)]NBMPR binding to intact erythrocytes and ghosts from human, hamster and mouse are summarized in Table 1. The binding curves (not shown) from which the binding parameters were derived indicated that both in human and mouse erythrocytes, the specific binding component represented about 80–90% of total binding throughout the entire concentration range tested. However, in hamster erythrocytes this component only was about 20%; accordingly, the values for hamster must be viewed with caution. In the case of ghost membrane studies, the specific binding represented 80–90% of total binding in all three species. Thus all data summarized in Table 1, with the possible exception of the parameters for hamster erythrocytes, represent meaningful estimates of affinity and maximal binding capacity.

The binding parameters for human and mouse erythrocytes are in the same order of magnitude, although mouse erythrocytes have about twice the number of binding sites per cell in comparison with the human. In hamster erythrocytes, the binding

Table 1. Binding parameters for [^{3}H(G)]NBMPR binding to erythrocytes and ghosts

Species	Erythrocytes*		Ghosts†	
	K_d (nM)	B_{max} (sites/cell)	K_d (nM)	B_{max} (pmol/mg)
Human	1.86 ± 0.41	3531 ± 409	3.34	1.20
Mouse	2.77 ± 0.27	8821 ± 612	3.72	1.19
Hamster	1.02 ± 0.41	674 ± 132	0.38	1.39

* Values are means ± SE, N = 3.
†Means of two experiments; individual values differing by less than 10%.

Table 2. IC_{50} Values for the inhibition of binding of [^{3}H(G)]NBMPR to intact erythrocytes from human, hamster and mouse by competing drugs

Competing drug	IC_{50} (μM)		
	Human	Hamster	Mouse
NMBPR	0.039 (0.030–0.050)	0.34 (0.30–0.40)	0.057 (0.040–0.080)
Dilazep	0.017 (0.015–0.020)	0.74 (0.060–0.100)	0.73 (0.06–0.11)
Soluflazine	0.18 (0.15–0.25)	78 (60–90)	76 (65–100)
Mioflazine	1.10 (1.00–1.20)	95 (80–110)	89 (80–100)
R57974	0.17 (0.15–0.20)	38 (35–40)	5.9 (5.5–6.5)

Geometric means (range) from three experiments.

affinity appears to be slightly higher than in human and mouse, but the number of binding sites per cell was found to be one order of magnitude below that found for human or mouse.

The binding affinities for [^{3}H(G)]NBMPR in ghost preparations from human and mouse were essentially identical, but in both cases were higher by a factor of about two as compared with intact cells. The binding capacity of hamster ghosts was the same as that of human and mouse ghosts, which contrasts with the finding of a much lower number of binding sites seen in intact cells (Table 1).

The binding studies with intact cells and isolated membranes revealed that the binding affinities of [^{3}H(G)]NBMPR in intact erythrocytes from the three species were in the same order of magnitude, thus it seemed warranted to perform comparative binding displacement studies with different potential competing drugs under the same conditions, i.e. a ligand concentration of 1 nM. Previous studies also have shown the dissociation constant of NBMPR to be in the order of 1 nM in mouse and human erythrocyte preparations [15, 20]. In the case of ghosts, however, the binding affinity of hamster membranes was about 10-fold higher than that of human or mouse membranes. However, for consistency 1 nM [^{3}H(G)]NBMPR was used with preparations from all three species.

Comparison of nucleoside transport inhibitor effects on adenosine transport and [^{3}H(G)]NMBPR binding in intact erythrocytes

The IC_{50} values obtained for various inhibitors with intact erythrocytes and isolated ghost membranes from human, mouse and hamster are summarized in Tables 2 and 3, respectively. The nucleoside transport inhibitory effects of NBMPR, dilazep and mioflazine derivatives have been studied in several systems including adenosine transport into isolated erythrocytes from various species [12], competition with [^{3}H(G)]NBMPR binding in isolated membranes from hamster tissues [13] and, presently, competition with [^{3}H(G)]NBMPR binding to isolated erythrocytes and corresponding ghost membranes. In order to facilitate a comparison, not only between data obtained in the present study but also those published earlier [12, 13], IC_{50} values from these different investigations have been normalized, using IC_{50} values of NBMPR as a reference, and summarized in Table 4. An absolute comparison, based on actual affinity data for individual ligands is not possible because only relative potency values have been obtained in each of the above mentioned studies. NBMPR was chosen as a reference compound because most studies in other systems have been obtained with this potent inhibitor and because it is most likely that this

Table 3. IC_{50} Values for the inhibition of binding of [^{3}H(G)]NBMPR to unsealed erythrocyte ghosts from human, hamster and mouse by competing ligands

Competing ligand	IC_{50} (μM)		
	Human	Hamster	Mouse
NBMPR	0.054 (0.040–0.080)	0.18 (0.15–0.20)	0.007 (0.006–0.008)
Dilazep	0.006 (0.005–0.007	0.70 (0.50–1.00)	0.18 (0.15–0.22)
Soluflazine	0.25 (0.20–0.28)	47 (40–60)	29 (25–35)
Mioflazine	0.04 (0.030–0.050)	47 (35–60)	12 (9–15)
R57974	0.009 (0.008–0.010)	1.28 (1.00–1.60)	0.18 (0.15–0.20)

Geometric means (range) from three experiments.

Table 4. Comparison of relative potencies of nucleoside transport inhibitors

Species	System	Relative potency of drug*				
		NBMPR	Dila	Mio	Solu	R57974
Human	Transport†	1	0.13	3.6	2.1	3.2
	Erythrocytes‡	1	0.43	28	4.6	4.4
	Ghosts‡	1	0.11	0.07	4.6	0.17
Mouse	Transport†	1	0.22	203	170	21
	Erythrocytes‡	1	12.8	1560	1330	103
	Ghosts‡	1	26	171	414	26
Hamster	Transport†	1	0.36	33	43	9.6
	Erythrocytes‡	1	2.2	280	230	112
	Ghosts‡	1	3.9	261	261	7.1
	Liver membranes§	1	80	36,400	11,400	930
	Lung membranes§	1	33	7600	16,900	57

* Relative potency values from different studies, setting IC_{50} value of NBMPR = 1.
† Data from Ref. 12.
‡ Data from present study, c.f. Tables 2 and 3.
§ Data from Ref. 13.

drug actually interacts directly with the transporter site, being a nucleoside derivative.

Firstly it is of interest to evaluate the relationship between potency values obtained in actual adenosine transport studies [12] and [^{3}H(G)]NBMPR binding studies in isolated, intact cells. It is immediately evident upon crude inspection (c.f. Table 4) that in each species different relative values or potency series have been observed, and accordingly each species has to be considered separately.

Human erythrocytes. There is a relatively good agreement between the potency series and the magnitude of the relative potency values in the human, with the exception of a poor effectiveness of mioflazine as an inhibitor of [^{3}H(G)]NBMPR binding. Dilazep was more potent than NBMPR both in transport studies and binding studies, and the other inhibitors, except as pointed out for mioflazine, were effective within the same order of magnitude of concentration. When comparing the present results of binding studies (erythrocytes and ghosts) with those of transport studies, much less agreement is apparent: While both dilazep and soluflazine show competition values similar to those seen in transport studies, both mioflazine and R57974 were about 20 and 30 times more effective in binding competition in ghosts than in intact cells. Thus the profile of drug competition with [^{3}H(G)]NBMPR binding in ghosts differs significantly both from that seen with inhibition of transport as well as inhibition of binding in intact cells.

Mouse erythrocytes. In the mouse, the major apparent difference is that dilazep proved much less effective in competition binding studies, both with intact cells and ghosts, than in previous transport studies. On the other hand, the values for mioflazine and its derivatives in all three systems show a common trend in relative potencies, although the magnitude of differences is considerably larger in the case of binding studies with intact cells, the three

mioflazine derivatives being five to ten times less potent in intact erythocytes than in membranes. A consistent finding, despite this difference in magnitude, is that R57974 proved considerably more potent than mioflazine or soluflazine in all three systems.

Hamster erythrocytes. In the hamster, dilazep also proved to be much less effective in binding studies than in transport studies. Further, the efficiency of all drugs competing for [^{3}H(G)]NBMPR binding was much reduced as compared to competing in adenosine transport studies, with the exception of R57974 which was an effective inhibitor in ghost membranes. In the case of the hamster a further comparison is possible between the results from various erythrocyte studies with previous ones from studies with membranes from liver and lung [13]. In the latter systems all inhibitors are much less potent then NBMPR, i.e. by up to four orders of magnitude in the case of mioflazine and soluflazine. A consistent feature is that among the mioflazine derivatives R57974 remained the most potent one in all systems, as it was also in the other species (exception: human transport studies).

Evaluation of nucleoside transport inhibitor effects in membranes

Keeping in mind the aim of the current study namely to assess whether transport inhibitors of different chemical types compete similarly for [^{3}H(G)]NBMPR binding to intact cells and to corresponding isolated membranes, it can be stated that intact cell and isolated membrane binding cannot be compared or related in a simple manner. As has been argued previously, based on [^{3}H(G)]NBMPR dissociation studies in the presence of dilazep, the binding site for this drug may not be the same as that for NBMPR or the permeation site for nucleosides on the membrane transporter [13]. Thus while dilazep very efficiently interferes with permeant transport in intact cells, it is less efficient as an inhibitor of NBMPR-binding in isolated membranes questioning whether the same site is involved or perhaps indicating that coupling to the transporter is disrupted upon cell breakage.

In isolated membranes, specific and nonspecific binding sites for ligands such as [^{3}H(G)]NBMPR may be uncovered on the inner membrane. In erythrocyte membranes two [^{3}H(G)]NBMPR photolabeled protein bands have been resolved, compared to only one band when intact cells were used [21]. This suggests that specific sites (possibly derived from the same nucleoside transporter) are uncovered in unsealed membranes. The binding characteristics of [^{3}H(G)]NBMPR or inhibitor drugs at the inner site(s) may differ from those of the outer site(s). It is also conceivable that competing ligands and drugs acting via allosteric mechanisms may be present at the outer site only or couple differently with the outer and inner sites, thus accounting for the widely differing competition values observed in different species and models.

Overall it appears that [^{3}H(G)]NBMPR binding and drug competition studies, both on the level of intact cells and isolated membranes, do not allow any predictions on whether or not different drugs are effective transport inhibitors. In turn, meaningful screening studies aimed at selecting suitable *in vivo* nucleoside transport inhibitors—and potential host protectors against cytotoxic nucleosides—preferably should be carried out with intact cells at the level of direct nucleoside transport studies.

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