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DIFFERENTIAL INHIBITION OF NUCLEOSIDE TRANSPORT SYSTEMS IN MAMMALIAN CELLS BY A NEW SERIES OF COMPOUNDS RELATED TO LIDOFLAZINE AND MIOFLAZINE

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Abstract—The sensitivity of facilitated-diffusion and Na^+-dependent nucleoside transporters to inhibition by a series of novel compounds related to lidoflazine and mioflazine was investigated. Uridine transport by rabbit erythrocytes, which proceeds solely by the nitrobenzylthioinosine (NBMPR)-sensitive facilitated-diffusion system, was inhibited with apparent K_i values of less than 10 nM by lidoflazine, mioflazine, soluflazine and R73-335. These compounds also blocked site-specific [^{3}H]NBMPR binding to rabbit erythrocyte membranes in a competitive fashion. The NBMPR-sensitive system in rat erythrocytes was also inhibited by lidoflazine, mioflazine, soluflazine and R73-335 but was two to three orders of magnitude less sensitive to inhibition than the system in rabbit erythrocytes (apparent K_i 7.3, 2.4, 5.7 and 0.1 μM, respectively). Lidoflazine, mioflazine and R73-335 exhibited a similar potency for the NBMPR-sensitive and -insensitive nucleoside transporters in rat erythrocytes. In contrast, soluflazine was 20- to 100-fold more potent as an inhibitor of the NBMPR-insensitive nucleoside transport component in rat erythrocytes (IC_{50} of 0.08–0.2 μM) compared to the NBMPR-sensitive nucleoside carrier in these cells ($IC_{50} \approx 10\,\mu$M). None of the test compounds were potent inhibitors of Na^+-dependent uridine transport in bovine renal brush-border membrane vesicles. These results indicate that lidoflazine, mioflazine, soluflazine and R73-335 are selective inhibitors of nucleoside transport in animal cells and that the potency of these compounds as nucleoside transport inhibitors is species dependent.

The passage of nucleosides and synthetic nucleoside analogues across the plasma membrane of animal cells may occur by simple diffusion or more frequently by carrier-mediated processes [1, 2]. Two types of facilitated-diffusion nucleoside transporters that differ in their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR†) have been recognized [3–6]. The most widely studied of these carriers is that found in many mammalian erythrocytes which is sensitive to inhibition by nanomolar concentrations (1–10 nM) of NBMPR (designated NBMPR-sensitive). Inhibition by NBMPR is associated with binding of the inhibitor to the cell membrane and a direct correlation between inhibition of transport and NBMPR binding has been demonstrated [7]. The other form, referred to as NBMPR-insensitive, is not associated with high-affinity binding sites and is inhibited only by micromolar concentrations (1–30 μM) of NBMPR. Both transporters accept a broad range of nucleosides as permeants but differ in their sensitivity to inhibition by extracellularly located *p*-chloromercuriphenyl sulphonate (PCMBS) [5, 6]. Recent studies on the isolation of nucleoside transport mutants [8] suggest the NBMPR-sensitive and -insensitive carriers are different gene products. In addition to these facilitated-diffusion carriers, active Na^+-dependent nucleoside transport systems have been described in renal and intestinal brush-border membrane vesicles, epithelial cells and mouse splenocytes [9–14]. To date, two Na^+-dependent nucleoside transporters have been delineated—one mainly specific for purine nucleosides and the other mainly specific for pyrimidine nucleosides [12–17]. However, uridine and adenosine appear to be permeants for both systems. NBMPR (3 μM) fails to inhibit either of the Na^+-nucleoside co-transporters [11, 14, 15, 17].

The existence of nucleoside transporters insensitive to inhibition by NBMPR has been exploited in chemotherapy strategies. For example, the co-administration of NBMPR with several cytotoxic nucleosides can dramatically increase the therapeutic index of the nucleoside drugs in the treatment of neoplasms and parasitic diseases, achieving cures in some instances [18, 19]. The basis of this selective protection of host tissues appears to be the insensitivity of the tumor cell or parasite transport system to NBMPR. Thus, there is much interest on whether other potential nucleoside transport inhibitors can discriminate between the different nucleoside transport systems. Recently a new group of drugs, mioflazine and its derivatives, have been suggested to possess nucleoside transport inhibitory activity in mammalian cells [20, 21]. However, the mechanism of action of these compounds, their relative potencies as inhibitors of nucleoside transport in isolated cells or membranes and their possible selectivity as inhibitors of the various nucleoside transporters are not known. In this study, we have investigated the ability

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† Abbreviations: NBMPR, nitrobenzylthioinosine, {6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine}; PCMBS, *p*-chloromercuriphenyl sulphonate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

of mioflazine and its derivatives to inhibit uridine transport by erythrocytes from a number of species, to inhibit Na^+-dependent uridine transport by renal brush-border membrane vesicles and to block [^{3}H]NBMPR binding. During the preparation of this manuscript the inhibitory effect of mioflazine and some of its derivatives on [^{3}H]NBMPR binding to membrane preparations from hamster tissues and calf lung tissue was reported [22, 23].

MATERIALS AND METHODS

Erythrocytes and membranes. Blood samples were collected into heparinized 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 a medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris–HCl (pH 7.4 at 22°), 2 mM $MgCl_2$ and 0.1 mM EDTA (disodium salt). For rat erythrocytes, the medium also contained 0.05% (w/v) BSA. Haemoglobin-free erythrocyte membranes were prepared as described previously and resuspended in 5 mM sodium phosphate (pH 7.4) [5].

Renal brush-border membrane vesicles. Bovine kidneys were obtained fresh from a local abattoir and placed on ice. The outer cortex was dissected, homogenized with a Polytron on setting 5 for 30 sec, repeated four times and brush-border membrane vesicles prepared by a Mg^{2+} precipitation technique [24]. The purity of the preparation was assessed by measuring the enrichment of the brush-border marker enzyme, alkaline phosphatase, as compared with the homogenate [25]. The enrichment on average was 10-fold. The vesicles were suspended in 250 mM sucrose, 0.2 mM $CaCl_2$ and 10 mM Hepes–Tris pH 7.4, frozen in liquid nitrogen and stored at −70°. Before use, the membrane suspension was revesiculated by passing the suspension 10 times through a 25 gauge needle.

Human placental brush-border membrane vesicles. Brush-border human placental membrane vesicles, representing the maternal facing surface of the syncytiotrophoblast, were prepared by the method of Glazier *et al.* [26]. The final vesicle preparation was resuspended in 5 mM Hepes–Tris (pH 7.4) and 300 mM mannitol, frozen in liquid nitrogen and stored at −70°. The brush-border suspension was on average enriched 18-fold with respect to the marker enzyme alkaline phosphatase as compared with the placental homogenate.

Nucleoside transport by erythrocytes. Uridine influx was measured at 22° by mixing 0.2 mL portions of cell suspension (haematocrit 10–20%) with 0.2 mL of medium containing the appropriate concentration of [^{3}H]uridine (2.5 μCi/mL). Inhibition studies were performed by one of two methods. In some studies, test compounds and radioactive nucleoside were added simultaneously. In other studies, the cell suspension was incubated with 1 mL of the test compound for 15 min. The cells were collected by centrifugation (10 sec, 12,000 *g*), 1 mL of medium removed and the cells resuspended in a further 1 mL of the test compound. After 15 min, the cells were pelleted, resuspended in a final volume of 0.2 mL and 0.2 mL of [^{3}H]uridine added to initiate the transport assay. At specified time intervals (30 and 4 sec for rat and rabbit erythrocytes, chosen to ensure initial rates of uridine influx were measured [5, 27]), cells were collected from incubation mixtures by one of two methods. For rat erythrocytes, a washing technique using ice-cold stop medium containing 50 μM dilazep was used as previously described [5]. For more rapid fluxes in rabbit erythrocytes, uridine influx was terminated by the "inhibitor-oil-stop" method [27]. Radioactivity associated with the cell pellet was determined as previously described [5, 27]. Transport rates were calculated after substraction of ^{3}H-activity which became associated with the cells due to non-mediated permeation. This correction was obtained by performing parallel experiments in the presence of 100 μM dipyridamole and 40 mM thymidine.

Nucleoside transport by membrane vesicles. The uptake of [^{3}H]uridine (20 μCi/mL) at 22° was measured using an inhibitor-stop filtration technique as described previously [10]. In inhibition studies, test compounds were preincubated with the renal brush-border membrane vesicles for 30 min before initiation of uridine transport. Na^+-dependent uridine influx was defined as that in the presence of a 100 mM NaCl gradient minus that in the presence of a 100 mM KCl gradient.

[^{3}H]*NBMPR binding.* Site-specific equilibrium binding of [^{3}H]NBMPR to membrane suspensions (0.03–0.3 mg of protein; total incubation volume 3 mL) was determined at 22° using rapid filtration through glass fibre filters (Whatman GF/B) to separate free inhibitor from membrane-bound inhibitor [17, 28]. For human placental membrane vesicles, the methodology was slightly modified to ensure 100% retention by the filter of the small vesicles. Whatman GF/B glass fibre filters were presoaked for at least 1 hr in 0.3% (v/v) polyethylenimine and placed on the filtration manifold with washing. Aliquots of the incubation were poured onto the filter and washed twice with 5 mL of ice-cold buffer. The filters were placed in scintillation vials with 4 mL of Optiphas T (LKB Scintillation Products), left overnight and counted for radioactivity.

Dissociation kinetics of [^{3}H]*NBMPR binding.* Rabbit erythrocyte membranes (final protein concn. 0.5 mg/mL) were incubated with [^{3}H]NBMPR (initial concn. 6.7 nM) in the presence and absence of 5 μM NBMPR for 30 min at 22°. Incubations were terminated by centrifugation at 45,000 *g* for 15 min and washing the membrane pellet once with ice-cold sodium phosphate buffer, pH 7.4. The final pellet was resuspended to a protein concentration of 1.5 mg/mL and kept on ice for periods of up to 6 hr. Dissociation of bound [^{3}H]NBMPR was initiated by adding 100 μL of labelled membranes to 60 mL of sodium phosphate buffer (22°) containing 1 μM of test compound. The medium was continuously stirred and 5 mL portions removed at various times (0.5–20 min) and filtered through glass-fibre filters (Whatman GF/B) which were washed with 5 mL of ice-cold buffer under suction. Radioactivity associated with the filters was measured as described above for [^{3}H]NBMPR binding. Non-specific [^{3}H]NBMPR binding was determined by performing the above procedure with the NBMPR-treated membranes and

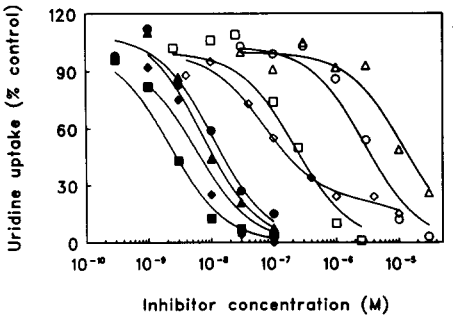

Fig. 1. Effect of mioflazine, lidoflazine, soluflazine and R73-335 on uridine influx by rabbit and rat erythrocytes. Cells (rabbit erythrocytes closed symbols, rat erythrocytes open symbols) were preincubated with mioflazine (●, ○), lidoflazine (▲, △), soluflazine (◆, ◇) and R73-335 (■, □) at 22° as described in Materials and Methods. Initial rates of [^{3}H]uridine uptake (0.2 and 0.1 mM for rabbit and rat erythrocytes, respectively) were determined using a 4 and 30 sec incubation interval for rabbit and rat erythrocytes, respectively. Results are given as a percentage of the control influx rate (28.8 and 1.1 mmol/L cells per hr for rabbit and rat erythrocytes, respectively). Values are the average of triplicate estimates.

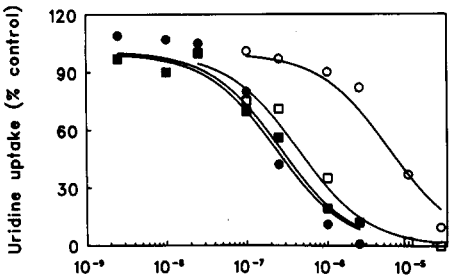

Fig. 2. Effect of soluflazine and R73-335 on uridine influx (0.1 mM) by NBMPR-treated and PCMBS-treated rat erythrocytes. Cells were pretreated with NBMPR (100 nM) at 22° for 30 min or PCMBS (200 μM) at 1° for 30 min and then washed twice to remove excess reagent. The treated erythrocytes were preincubated with soluflazine (●, ○) or R73-335 (■, □) as described in Materials and Methods. Values are the average of triplicate estimates and expressed as percentages of the uridine transport rate in the absence of inhibitor. The control transport rates were 0.65 and 0.16 mmol/L. cells per hr for NBMPR-treated (closed symbols) and PCMBS-treated (open symbols) erythrocytes.

typically was less than 3% of the total binding. Specific binding was defined as the difference between total binding and non-specific binding. The dissociation rate constant, k_{-1}, was determined using the following equation:

$$\ln \frac{B_t}{B_e} = -k_{-1}.t \qquad (1)$$

where B_e is the specific binding at time zero and B_t is the specific binding at time t.

Data analysis. The experimental data of the competition studies were fitted to the mathematical expression describing the appropriate curve for either a single-site model or a two-site model using

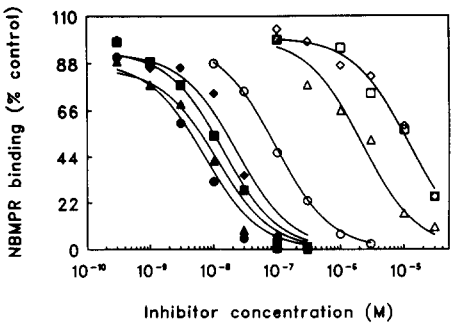

Fig. 3. Effect of mioflazine, lidoflazine, soluflazine and R73-335 on [^{3}H]NBMPR binding to rabbit and rat erythrocyte membranes. Site-specific (sensitive to 10 μM NBMPR) [^{3}H]NBMPR binding to rabbit (closed symbols) and rat (open symbols) erythrocyte membranes were measured in the presence of R73-335 (●, ○), mioflazine (▲, △), soluflazine (■, □) and lidoflazine (◆, ◇). Results are plotted as a percentage of control high-affinity NBMPR binding activity in the absence of inhibitors (13.2 and 0.13 pmol/mg protein for rabbit and rat erythrocyte membranes). Values are the means of triplicate estimates and [^{3}H]NBMPR concentrations were chosen to be close to the apparent K_d value for [^{3}H]NBMPR binding. [^{3}H]NBMPR concentrations were 1.0 and 0.1 nM for rabbit and rat erythrocyte membranes, respectively.

the computer program "fit-curve" [29]. The curves in Figs 1–3 are the "best-fit" computer curves.

Chemicals. [G-^{3}H]NBMPR (23 Ci/mmol) and [5,6-^{3}H]uridine (40 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA, U.S.A.) and New England Nuclear (Stevenage, U.K.), respectively. NBMPR, dipyridamole and phloridzin were obtained from the Sigma Chemical Co. (Poole, U.K.). Dilazep was a generous gift from Hoffman-La Roche and Co. (Basel, Switzerland). Lidoflazine, mioflazine, soluflazine and R73-335 were gifts from Dr H. Van Belle, Janssen Pharmaceutica, B-2340 Beerse, Belgium. Stock solutions (2 mM) of these compounds were dissolved in dimethyl sulphoxide from which they were diluted into aqueous buffers to the appropriate concentration. Where necessary control solutions also contained dimethyl sulphoxide. All other reagents were of analytical grade.

RESULTS

Uridine transport

Preliminary studies demonstrated that preincubation of rabbit erythrocytes with the inhibitors compared to simultaneous addition of the inhibitor and [^{3}H]uridine resulted in a 100-fold increase in apparent potency of the test compound (data not shown). Thus, in future transport inhibition studies, erythrocytes and membrane vesicles were preincubated with the transport inhibitor as described under Materials and Methods.

The dose–response curves for inhibition of uridine influx in rabbit and rat erythrocytes by mioflazine, soluflazine, lidoflazine and R73-335 are compared in Fig. 1. All the compounds were potent inhibitors of uridine influx by rabbit erythrocytes with IC_{50} values

Table 1. Comparison of the potencies of mioflazine, soluflazine, lidoflazine and R73-335 for the inhibition of uridine influx and NBMPR binding by rat and rabbit erythrocytes

Species inhibitor	Uridine influx		NBMPR binding	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
Rat				
Lidoflazine	5 ± 2.5	ND	11.2 ± 1.8	7.3 ± 1.2
Mioflazine	2.3 ± 0.35	ND	3.6 ± 1.0	2.4 ± 0.7
Soluflazine	0.084 ± 0.035 and 10.6 ± 8.5	ND	8.7 ± 1.9	5.7 ± 1.3
R73-335	0.14 ± 0.04	ND	0.15 ± 0.07	0.10 ± 0.048
Rabbit				
Lidoflazine	0.010 ± 0.001	0.0047 ± 0.004	0.023 ± 0.003	0.014 ± 0.002
Mioflazine	0.018 ± 0.003	0.0084 ± 0.0014	0.0081 ± 0.002	0.0051 ± 0.0013
Soluflazine	0.010 ± 0.006	0.0046 ± 0.0028	0.012 ± 0.001	0.0077 ± 0.0007
R73-335	0.008 ± 0.003	0.0036 ± 0.0012	0.0046 ± 0.0015	0.0029 ± 0.001

Uridine influx (0.1 and 0.2 mM for rat and rabbit erythrocytes) and site-specific [^{3}H]NBMPR binding (0.1 and 1.0 nM for rat and rabbit erythrocyte ghosts) were determined as described in the text. IC_{50} values were determined from dose–response curves. Where appropriate K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_m$ or $K_d)$ where K_m for uridine influx by rabbit erythrocyte was taken as 0.17 mM [27] and K_d values for NBMPR binding by rat and rabbit erythrocyte ghosts were determined as 0.19 and 1.7 nM, respectively. The values are the average of at least three separate experiments ± SE.
ND, not determined.

of 2.2–11 nM. In contrast, mioflazine and its derivatives were 10- to 200-fold less potent as inhibitors of uridine transport by rat erythrocytes. Moreover, it is noticeable that the dose–response curve for soluflazine is shallow with a Hill coefficient of 0.60 ± 0.04 (mean ± SE (3)) suggesting the presence of two separate components (see below). The mean IC_{50} values from at least three experiments are given in Table 1. For soluflazine inhibition of uridine uptake by rat erythrocytes, the data was best fitted to a two-site model (see Materials and Methods). On average 67% of the uridine flux by rat erythrocytes at 0.1 mM was inhibited by soluflazine with a relative high affinity (mean IC_{50} value for three experiments of 0.084 ± 0.035 μM).

Rat erythrocytes have been demonstrated previously to transport nucleosides by two separate facilitated-diffusion routes [4, 5]. It thus seemed likely that, in view of the shallow dose–response curve for soluflazine (Fig. 1), soluflazine was able to discriminate between the two transport components in rat erythrocytes. This was tested directly by using rat erythrocytes pretreated with either NBMPR (100 nM) or PCMBS (200 μM) to selectively block the NBMPR-sensitive and -insensitive transport systems, respectively [5]. Figure 2 shows the results of such an experiment where the dose–response curves for soluflazine and R73-335 are compared for the NBMPR-sensitive and -insensitive components. Soluflazine exhibited a 14-fold difference in the IC_{50} value for the two systems, 0.2 and 7 μM, respectively, for the NBMPR-insensitive and -sensitive transporters. In contrast, the IC_{50} values for R73-335 inhibition are similar for the two carriers. Using the previously reported K_m values for uridine influx by the NBMPR-sensitive and -insensitive routes (50 and 163 μM, respectively) [5], the apparent K_i values determined from these IC_{50} values by the NBMPR-sensitive and -insensitive transporters were, respectively, 2.3 and 0.12 μM for soluflazine and 170 and 170 μM for R73-335.

NBMPR binding

Analysis of the kinetics of transport inhibition was not possible by conventional methods in which the inhibitor and permeant are added simultaneously due to the problems noted above. However, the NBMPR-sensitive nucleoside transporter can also be investigated by studying the properties of high-affinity NBMPR binding. The effect of varying concentrations of mioflazine, soluflazine, lidoflazine and R73-335 on site-specific binding of NBMPR to rabbit and rat erythrocyte membranes is shown in Fig. 3. Consistent with the transport inhibition data (Figs 1 and 2; Table 1), the transport inhibitors were more potent at blocking [^{3}H]NBMPR binding to rabbit erythrocyte membranes compared to rat erythrocyte membranes. In other experiments, the nature of the inhibition by mioflazine, soluflazine, lidoflazine and R73-335 was explored by using various concentrations of the transport inhibitor and [^{3}H]NBMPR (Fig. 4 shows the data for lidoflazine and soluflazine). For all four compounds the inhibitor profiles for rabbit erythrocyte membranes were consistent with a competitive type of inhibition, with apparent K_i values of 4, 6, 14 and 3.5 nM for mioflazine, soluflazine, lidoflazine and R73-335, respectively. Mioflazine and its derivatives were also shown to inhibit specific binding of NBMPR to human placental brush-border membrane vesicles, a preparation that exhibits NBMPR-sensitive adenosine uptake [30], with the same order of potency as observed in rabbit erythrocyte membranes but with slightly increased IC_{50} values (mean IC_{50} values ± SE for three different preparations of 94 ± 15, 78 ± 36, 17 ± 2 and 2.3 ± 0.4 nM for lidoflazine, soluflazine, mioflazine and R73-335, respectively).

In other experiments, the effect of 1 μM mioflazine and its derivatives on NBMPR dissociation from rabbit erythrocyte membranes was examined. The apparent dissociation rate constants (mean ± SE) for three separate determinations were 0.62 ± 0.09,

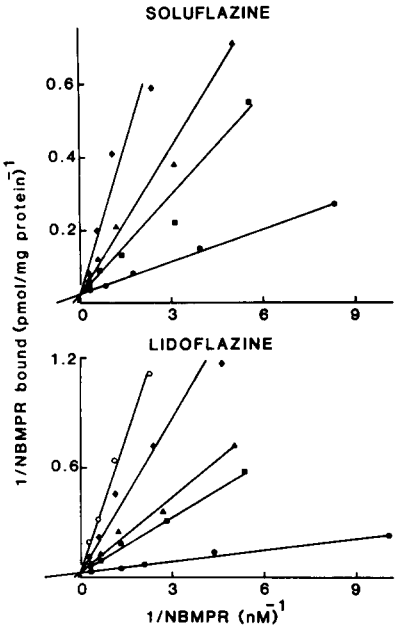

Fig. 4. Effect of lidoflazine and soluflazine on equilibrium [^{3}H]NBMPR binding to rabbit erythrocyte membranes. The reciprocals of [^{3}H]NBMPR bound to high-affinity sites in the presence of various concentrations of lidoflazine and soluflazine at 22° are plotted against the respective reciprocals of the free equilibrium concentrations of [^{3}H]NBMPR. Concentrations (nM) of soluflazine were 0 (●), 10 (■), 20 (▲) and 40 (◆), and for lidoflazine, 0 (●), 20 (■), 40 (▲), 80 (◆) and 160 (○). Apparent K_i values, estimated from a plot of apparent K_d values versus inhibitor concentrations, were 14 and 6 nM for lidoflazine and soluflazine, respectively.

0.53 ± 0.04, 0.55 ± 0.05, 0.56 ± 0.03 and $0.60 \pm 0.03\ min^{-1}$ for soluflazine, lidoflazine, mioflazine, R73-335 and NBMPR, respectively. In contrast, dilazep (1 μM) inhibited the dissociation of bound [^{3}H]NBMPR (apparent dissociation constant $0.16 \pm 0.01\ min^{-1}$).

Na^+-*dependent uridine transport*

In bovine renal brush-border membrane vesicles uridine is transported, not by a facilitated-diffusion system, but by two Na^+-dependent carriers [16, 17]. Table 2 demonstrates that concentrations of mioflazine, lidoflazine, soluflazine and R73-335 as high as 34 μM had no significant effect on Na^+-dependent uridine influx in bovine renal brush-border membrane vesicles.

DISCUSSION

The experiments described in this paper show that lidoflazine, mioflazine and derivatives of these compounds are potent and selective inhibitors of the various types of nucleoside transport proteins that exist in mammalian cells. In particular, facilitated-diffusion nucleoside transporters are inhibited but Na^+-dependent uridine transport is resistant to inhibition. Furthermore, there exist clear differences in the sensitivity of the nucleoside transporters to inhibition by these compounds that are species related.

Lidoflazine, mioflazine, soluflazine and R73-335 were all potent inhibitors of the NBMPR-sensitive facilitated-diffusion nucleoside carrier in rabbit erythrocytes as demonstrated from both direct inhibition studies of uridine influx and by the ability of the compounds to block NBMPR binding in a competitive manner (apparent K_i values 2.9 to 14 nM). Furthermore, mioflazine and its derivatives had no effect on the dissociation of [^{3}H]NBMPR from its high-affinity binding site. A previous report has also shown that lidoflazine has no effect on the rate of [^{3}H]NBMPR dissociation from human erythrocytes [31]. However, as demonstrated previously for human erythrocytes and a variety of cultured cell lines [32, 33], dilazep inhibited the dissociation of [^{3}H]NBMPR from rabbit erythrocyte membranes. Taken together these results suggest that lidoflazine, mioflazine, soluflazine and R73-335 interact specifically with the NBMPR-sensitive transporter at a common or overlapping site in rabbit erythrocytes. This site or part of it is also proposed to accommodate NBMPR.

In other species and tissues the potencies of mioflazine and its derivatives as inhibitors of the NBMPR-sensitive nucleoside carrier was reduced compared to rabbit erythrocytes. For human placental brush-border membrane vesicles an average reduction in potency of 5-fold was observed with the exception of R73-335 which had a similar potency to that demonstrated in rabbit erythrocytes. However, the NBMPR-sensitive nucleoside carrier in rat erythrocytes was two to three orders of magnitude less sensitive to inhibition than the transporter in rabbit red blood cells. The resistance of the NBMPR-sensitive carrier in rat erythrocytes to mioflazine and its derivatives was not unexpected as previous work has noted the low potency of dipyridamole, dilazep and lidoflazine as inhibitors of nucleoside transport activity in rat tissues [5, 34]. In a recent report [35] mouse and hamster erythrocytes were also shown to be resistant to inhibition by mioflazine and some of its derivatives (IC_{50} values > 200 nM). These data suggest that rodent tissues are particularly resistant to inhibition by this group of compounds despite the observation that the equilibrium binding affinity for [^{3}H]NBMPR for these species are in the same order of magnitude [1, 2, 27, 35]. It is possible to envisage a small structural or conformation change in the rat, mouse and hamster transporter which selectively decreases its affinity for mioflazine and its derivatives without an effect on NBMPR.

The second type of facilitated-diffusion nucleoside transporter present on some cell types is the NBMPR-insensitive system [1–6, 8]. Rat erythrocytes possess both the NBMPR-sensitive and -insensitive systems [4, 5]. Both transporters in rat erythrocytes appeared to be equally resistant to inhibition by lidoflazine, mioflazine and R73-335 as judged by the monophasic inhibition curves presented in Fig. 1. Similar results have been obtained for dipyridamole inhibition [5]. In contrast, soluflazine exhibited a shallow dose–response curve that was consistent with two components. Indeed,

Table 2. Effect of mioflazine, lidoflazine, soluflazine and R73-335 on Na^+-dependent uridine uptake by bovine renal brush-border membrane vesicles

Inhibitor concn. (μM)	Na^+-dependent uridine influx (% control) 3.4	16.8	34
Soluflazine	99 ± 12	94 ± 11	104 ± 5
Lidoflazine	94 ± 9	98 ± 4	104 ± 2
Mioflazine	85 ± 3	92 ± 4	94 ± 5
R73-335	79 ± 8	83 ± 17	95 ± 4

The uptake of 10 μM uridine (3 sec) was initiated by addition of bovine renal brush-border membrane vesicles, preincubated with test compound for 30 min, to medium containing (final concn.) 10 μM [^{3}H]uridine, 100 mM NaCl or 100 mM KCl, 167 mM sucrose and 10 mM Hepes–Tris, pH 7.4. Results are the means of triplicate estimates expressed as a percentage of the control flux (uptake in the presence of NaCl minus that in the presence of KCl; mean ± SD).

direct transport measurements on each separate component of nucleoside influx in rat erythrocytes demonstrated that the NBMPR-insensitive nucleoside transporter is 20- to 100-fold more sensitive to inhibition by soluflazine than the NBMPR-sensitive transport system. Further studies are required to establish whether soluflazine is also able to discriminate between the NBMPR-sensitive and -insensitive nucleoside transporters in cells and tissues not derived from rat.

The usefulness of the displacement of [^{3}H]NBMPR binding as a means of predicting the potency of mioflazine and its derivatives as nucleoside transport inhibitors has recently been challenged due to discrepant data between inhibition of [^{3}H]NBMPR binding and nucleoside transport [35]. However, in the present study close agreement was observed between the apparent K_i values for inhibition of [^{3}H]NBMPR binding and nucleoside influx (Table 1). We have taken particular care to employ experimental protocols that minimize the depletion of free concentration of the inhibitor that may occur by specific and non-specific mechanisms and to allow sufficient time for the inhibitors to bind and exert maximal inhibition. In the earlier study [35], these concerns were not addressed and this probably explains the discrepancy in the values reported between inhibition of [^{3}H]NBMPR binding and nucleoside transport. Of course, [^{3}H]NBMPR binding will provide no information on the NBMPR-insensitive and Na^+-dependent nucleoside transport systems. For these carriers direct transport studies are needed.

In conclusion, mioflazine, lidoflazine, soluflazine and R73-335 are selective inhibitors of the nucleoside transporters in animal cells. The potency of these compounds as nucleoside transport inhibitors is species related—rat cells being particularly resistant to inhibition. However, soluflazine is a more potent inhibitor of the NBMPR-insensitive nucleoside transporter in rat erythrocytes compared to the NBMPR-sensitive nucleoside transport system in these cells. Further studies are required to test whether such selectivity with soluflazine is also observed in cells derived from species other than rat.

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