J. Pharm. Pharmacol. 1990, 42: 367–369
Communicated November 5, 1989

Potencies of mioflazine and its derivatives as inhibitors of adenosine transport in isolated erythrocytes from different species

HANS P. BAER, AFROZUL HAQ, ALI EL-SOOFI, VINCENT SERIGNESE, ALAN R. P. PATERSON*, *Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Centre, Box 3354, Riyadh 11211, Saudi Arabia and * Cancer Research Unit, Faculty of Medicine, University of Alberta, Edmonton T6G 2H7, Canada*

Abstract—The potency of mioflazine and related drugs (Janssen Pharmaceutica, Belgium) as inhibitors of adenosine transport in isolated erythrocytes from several species were measured and compared with those of dilazep and 6-(4-nitrobenzylmercapto)purine ribonucleoside (NBMPR). [8-^{3}H]Adenosine was used as the permeant at 1 μM and incubation times were 10 s, and assays were conducted in the presence and absence of varying doses of potential transport inhibitors. The species investigated included mouse, hamster, rabbit, baboon and man. Dilazep was the most potent compound throughout with an IC50 of about 2 nM. In the mouse and hamster mioflazine and its derivatives were considerably less potent (IC50 values > 200 nM) with the exception of R57974 with IC50 values of about 150 and 60 nM in mouse and hamster, respectively. In the man and baboon the derivatives had IC50 values in the same order of magnitude as NBMPR (< 100 nM), and in the rabbit they had potencies close to that of NBMPR, ranging between 10–60 nM. Nucleoside transport inhibitors are of potential importance as host protectors during treatment of parasitic infections with cytotoxic nucleosides. Present data indicate that mioflazine and its derivatives are not very potent in some of the preferred animal models for parasitic infections (mouse, hamster) but are more effective in primates such as man and baboon.

Nucleoside transport into mammalian cells mainly occurs via a facilitated, carrier-mediated process (cf. Paterson et al 1981) which is inhibitable by a number of drugs including nucleoside derivatives such as NBMPR and structurally diverse compounds with vasodilator properties (cf. Paterson et al 1981, 1983a, b; Hammond et al 1983). Interference with nucleoside transport in animals by administration of these type of drugs will enhance or prolong the physiological or pharmacological actions of adenosine, the presumed physiological permeant of nucleoside transporter systems, causing vasodilation, cardiac and CNS depression as well as promoting sleep (cf. Berne et al 1983). Nucleoside transport inhibitors also have been used in combination with cytotoxic nucleosides in the experimental treatment of parasitic infections, exploiting the differential sensitivity of the transport systems to inhibitors in mammalian cells and parasitic organisms (El Kouni et al 1983; cf. Baer 1989).

Recently a new group of drugs, mioflazine and its derivatives, has been shown to possess nucleoside transport inhibitory activity in mammalian cells (Van Belle et al 1986a, b). These compounds were suggested to possess potencies similar to NBMPR and to be orally applicable (Van Belle, personal communication). In order to explore the usefulness of these new drugs for potential therapeutic applications, we investigated their relative potencies as adenosine transport inhibitors in isolated erythrocytes, comparing their effects with those of NBMPR and dilazep. Considerable differences were observed in the relative potency profiles using erythrocytes from different species. Preliminary data from this study have been communicated (Baer & Serignese 1988; Baer et al 1988).

Correspondence to: H. P. Baer, Biological and Medical Research, King Faival Research Centre, Box 3354, Riyadh 11211, Saudi Arabia.

Materials and methods

Cell preparation. Blood was collected into heparinized vacutainer tubes from Balb/c mice, New Zealand White rabbits, Syrian-Golden hamsters, a male baboon and volunteers. After centrifugation (1000 *g*, 5 min), cells were washed three times with a 20-fold volume of phosphate-buffered saline (PBS, 0·9 NaCl, 10 mM sodium phosphate, pH 7·4) with removal of the buffy coats. Washed erythrocytes were suspended in a transport medium containing bicarbonate-free Eagle's Basal medium buffered with 20 mM HEPES, pH 7·4. Cell density was adjusted to about $2{\cdot}5 \times 10^9$ cells mL^{-1}. Cell counts were performed by haemocytometer. Cells were used for transport assays within 1 h of isolation.

Transport assay. Adenosine transport measurements were performed essentially as described by Paterson et al (1983b, 1985) with minor modifications. All component solutions were prepared in transport medium and stored frozen. Unless otherwise indicated, serial drug dilutions and the transport assay were performed by a TECAN robotic sampler providing for optimal volumetric control and timing. Assays were performed in 1·5 mL Eppendorf polypropylene tubes by mixing 100 μL 2 μM [8-^{3}H]adenosine (120 000–140 000 d min^{-1}) with 100 μL erythrocytes ($2{\cdot}5 \times 10^7$ cells). After 10 s reaction time a stopping solution of 200 μL 1 mM dilazep was added, followed within 15–30 s by manual addition of 400 μL dibutylphthalate and centrifugation for 15 s at 10 000 *g*, thus pelleting the cells under the oil layer. The reactions were carried out at ambient temperature (22°C). Drug dilutions (nucleoside transport inhibitors) in the assays ranged between 10^4 to 10^{-2} nM, in 1 : 3 serial dilutions.

Following centrifugation, the medium above the oily dibutylphthalate layer was removed by suction, followed by washes with H_2O. Then the oil layer was also aspirated, to leave the cell pellet undisturbed. The pelleted erythrocytes were lysed by mixing with 100 μL 1% Triton X-100. After the protein had been precipitated with 1 mL 5% trichloroacetic acid, the caps of the tubes were cut off and the entire tubes plus contents placed into scintillation vials. After the addition of 15 mL of scintillation cocktail (Optifluor, Amersham) with thorough mixing, radioactivity was measured in a liquid scintillation counter. This procedure avoided the hazardous transfer of radioactive liquid from Eppendorf tubes to scintillation vials.

Liquid scintillation counting was performed with quench correction to obtain absolute count rates. Based on these, the cell numbers in assays and the specific radioactivity of the adenosine (which was determined within each experiment) transport rates were calculated and expressed as "mol adenosine per cell per second". The relative potencies of nucleoside transport inhibitors were obtained from plots of transport rates versus log [inhibitor concentration] by interpolating the concentrations of drugs causing half-maximal inhibition (IC50).

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-(fluorophenyl)-4-(3-pyridinyl)-butyl]-*N*-(2,6-dichlorophenyl)-1-piperazineacetamide 2HCl) and R57974 (2-(aminocarbonyl) - 4 - [5,5 - bis(4 - fluorophenyl)pentyl-*N* - (2,6-dichlorophenyl)-1-piperazineacetamide HCl) 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). 6-(4-Nitrobenzyl)mercaptopurine (NBMPR) was a gift from Dr A. R. P. Paterson (Cancer Research Unit, University of Alberta, Edmonton, Canada). [8-^{3}H]Adenosine (31 Ci mmol^{-1}) was purchased from Moravec Biochemicals (Brea, CA, USA).

Mioflazine, soluflazine, NBMPR and R57974 were dissolved in dimethyl sulphoxide at a concentration of 10^{-2} M, and rapid dilutions into 0·9% NaCl (saline) warmed to about 40°C were made to obtain stock solutions of 10^{-5} M. Dilazep was well soluble in water or saline, or in transport medium, to prepare necessary stock solutions including a stopping solution with 1 mM dilazep.

Results

In initial studies with mouse erythrocytes it was established that allowing 10 s for transport assays still covered the near-linear range for the transport rate curve, with about 10% deviation from linearity occurring after about 20–25 s. Assays in the presence of inhibitors were then conducted with erythrocyte preparations from different species. Representative transport competition curves for mouse and human erythrocytes are shown in Fig. 1. There was no indication, in repeated experiments, of the existence of any biphasic displacement curves, suggesting the possibility of high and low affinity binding sites for transport inhibitors, as observed by Jarvis & Young (1986) in the case of NBMPR and its inhibition of uridine transport in rat

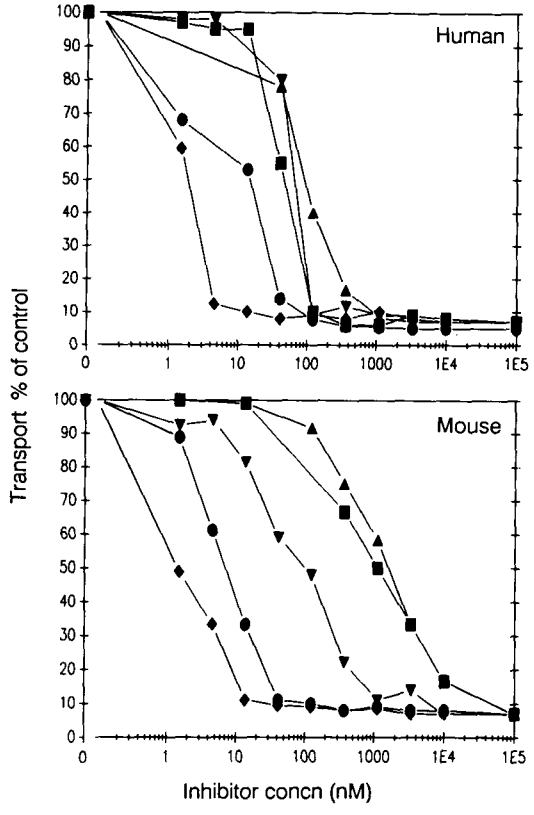

FIG. 1. Representative competition curves with various nucleoside transport inhibitors in isolated erythrocytes from man and mouse. Drugs included NBMPR (●), dilazep (◆), mioflazine (▲), soluflazine (■) and R57974 (▼). Error bars have been omitted for clarity; standard errors were below 10% of values.

Table 1. IC50 values of transport inhibitors in different species.

	Transport inhibitor IC50 (nM)*				
Species	NBMPR	Mioflazine	Soluflazine	R57974	Dilazep
Man	19·6 (16·3–23·7)* n=4	71·5 (48·7–104·8) n=5	41·7 (36·3–48·0) n=5	63·5 (44·1–91·3) n=5	2·7 (0·5–14·3) n=5
Mouse	7·4 (6·0–8·9) n=7	1506 (838–2705) n=3	1260 (945–1679) n=4	153 (63–372) n=4	1·7 (0·4–6·7) n=3
Rabbit	57·4 (50·7–65·8) n=5	42·6 (28·2–63·0) n=4	10·8 (3·5–32·5) n=4	52·3 (13·8–246) n=4	4·7 (2·1–10·0) n=4
Hamster	6·1 (5·6–6·6) n=6	205 (111–379) n=4	262 (105–650) n=3	58·5 (139·0–246) n=3	2·2 (0·8–5·8) n=3
Baboon	13·3 (12·6–14·0) n=3	54·7 (30·7–97·7) n=2	73·6 (38·1–142) n=3	84·3 (58·4–121·6) n=3	1·9 (0·4–9·8) n=2

* Means and 95% confidence limits.
n = number of experiments, except baboon where n = number of repetitions from one animal.

erythrocytes. The unexpected large differences in the relative potencies of the tested drugs in mouse and human caused us to expand this study to other species.

The *IC50* values for transport inhibitors in the different species studied are summarized in Table 1. At least three experiments, with blood samples from different animals or volunteers, were carried out; however, in the case of baboon only one animal was available to us, and experimental results from two or three blood samplings from the same animal are listed in the Table. The control transport rates in erythrocytes before addition of inhibitors were (means of 2–7 experiments; mol $cell^{-1}\ s^{-1} \times 10^{-20}$): 2·0 (man), 0·85 (mouse), 1·10 (rabbit), 0·86 (hamster) and 2·5 (baboon). The extent of maximal transport inhibition in the presence of 0·3 mM dilazep was better than 90% in all species.

Among the different species tested, dilazep always showed the highest potency followed by NBMPR, except in the rabbit where NBMPR was slightly less potent than soluflazine. Among the mioflazine derivatives, R57974 was most potent in the mouse and hamster. There was no significant difference between the mioflazine derivatives in the man, the rabbit or the baboon. In man and the baboon, the mioflazine derivatives showed a potency only slightly below that of NBMPR, and in the rabbit they were essentially equipotent with NBMPR. In the mouse mioflazine and soluflazine were very poor inhibitors with potencies more than 100-fold below that of NBMPR. R57974 was significantly more potent than mioflazine or soluflazine in mouse cells, but still about 20 times less potent than NBMPR.

Although absolute comparisons between potencies for a given drug among different species have to be made with caution considering the possibility of significant differences in the transport rate curves (K_m) (Jarvis et al 1982), it is interesting to observe that dilazep had essentially the same apparent IC50 value in all species.

Discussion

The mioflazine derivatives unexpectedly showed poor potency as nucleoside transport inhibitors in mouse erythrocytes compared with NBMPR and dilazep. This result contrasted with previous findings suggesting that these compounds were as effective as NBMPR in man or dog (Van Belle, personal communication). Thus other species, including man, were studied revealing that the relative potencies of transport inhibitors in different species are very variable.

Mioflazine or its derivatives thus appear to be poor choices for experimental work requiring effective in-vivo nucleoside transport inhibition in the hamster and especially the mouse (compound R57974 being a possible exception in the hamster). However, they appear to be promising in man and baboon as well as in the rabbit. In fact, in the latter species soluflazine even showed a potency significantly higher than NBMPR.

An absolute comparison of IC50 values from different species for the drugs studied in this investigation has to take into account the possibility that the binding affinity of NBMPR (K_m) differs among species. Jarvis et al (1982), using uridine as a permeant, found that these differences in K_m were small between several species including mouse, rabbit and man. It is tempting to consider that the consistently low IC50 values for dilazep, showing that it was the most potent compound throughout, reflect the existence of a separate binding site and mechanism of action (cf. Koren et al 1983). The binding site for mioflazine, on the other hand, appears to be distinct from that for dilazep or NBMPR since its characteristics (IC50) show considerable variation among the species tested.

We conclude that the potency of a given nucleoside transport inhibitor for experimental purposes and potential clinical development has to be established individually in each species and cannot be predicted from studies in an other. In addition, it is to be expected that pharmacokinetic parameters will be variable among species so that drug potency alone does not remain a sufficient or exclusive selection criterion.

The generous support provided for this study by Sheikh Rafiq El-Hariri is acknowledged most gratefully.

References

Baer, H. P. (1989) Cytotoxic nucleosides and parasitic diseases: A new therapeutic approach. Ann. Saudi Med. 9: 570–575

Baer, H. P., Serignese, V. (1988) Relative potencies of some adenosine transport inhibitors in mouse and human erythrocytes. Med. Sci. Res. 16: 933–934

Baer, H. P., Soofi, A., Serignese, V., Selim, A., Arfaa, A., Mahboubi, E. (1988) Search for nucleoside transport inhibitors for use in combination treatment with cytotoxic nucleosides of schistosomiasis and other parasitic disease. XIIth Intl. Congr. Tropical Medicine and Malaria, Amsterdam, Abstracts

Berne, R. M., Rall, T. W., Rubio, R. (1983) Regulatory function of adenosine. The Hague, Boston, London: Martinus Nijhoff Publishers

El Kouni, M. H., Diop, D., Cha, S. (1983) Combination therapy of schistosomiasis by tubercidin and nitrobenzylthioinosine 5′-monophosphate. Proc. Nat. Acad. Sci. USA 80: 6667–6670

Hammond, J. R., Jarvis, S. M., Paterson, A. R. P., Clanachan, A. S. (1983) Benzodiazepine inhibition of nucleoside transport in human erythrocytes. Biochem. Pharmacol. 32: 1229–1235

Jarvis, S. M., Hammond, J. R. Patterson, A.R.P., Clanachan, A.S. (1982) Species differences in nucleoside transport: a study of uridine transport and nitrobenzylthioinosine binding by mammalian erythrocytes. Biochem. J. 208: 83–88

Jarvis, S. M., Young, J. D. (1986) Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and p-chloromercuryphenyl sulfonate. J. Membr. Biol. 93: 1–10

Koren, R., Cass, C. E., Paterson, A. R. P. (1983) The kinetics of the dissociation of the inhibitor of nucleoside transport, nitrobenzylthioinosine, from the high affinity binding sites of cultured hamster cells. Biochem. J. 216: 299–308

Paterson, A. R. P., Kolassa, N., Cass, C. E. (1981) Transport of nucleoside drugs in animal cells. Pharmacol. Ther. 12: 515–536

Paterson, A. R. P., Jakobs, E. S., Harley, E. R., Cass, C. E., Robins, M. J. (1983a) Inhibitors of nucleoside transport as probes and drugs. In: Cheng, Y. C. (ed.) Development of target-orientated anticancer drugs. Raven Press, New York, pp 41–56

Paterson, A. R. P., Jakobs, E. S., Harley, E. R., Fu, N. W., Robins, M. J., Cass, C. E. (1983b) Inhibition of nucleoside transport. In Berne, R. M., Rall, T. W., Rubio, R. (eds) Regulatory function of adenosine. Martinus Nijhoff Publishers: The Hague/Boston/London, pp 203–220

Paterson, A. R. P., Harley, E. R., Cass, C. E. (1985) Measurement and inhibition of membrane transport of adenosine. Methods in Pharmacology 6: 165–180

Van Belle, H., Wynants J., Xhonneux, R., Flameng, W. (1986a) Changes in creatine phosphate, inorganic phosphate and the purine pattern in dog hearts with time of coronary artery occlusion and effect thereon of mioflazine, a nucleoside transport inhibitor. Cardiovasc. Res. 20: 658–664

Van Belle, H., Xhonneux, R., Flameng, W., Wynants, J. (1986b) Oral pretreatment with mioflazine completely changes the pattern and remarkably prolongs the accumulation of nucleosides in ischemic and reperfused myocardium. Basic Res. Cardiol. 81: 407–416