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BINDING OF [G-^{3}H]6-(4-NITROBENZYLMERCAPTO)PURINE RIBONUCLEOSIDE TO ISOLATED MEMBRANES

INHIBITORY EFFECT OF MIOFLAZINE AND ITS DERIVATIVES

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Abstract—The binding of [G-^{3}H]-6-(4-nitrobenzylmercapto)purine ribonucleoside ([G-^{3}H]NBMPR) was investigated using a centrifugation assay with membrane preparations from hamster tissues including liver, lung, kidney and heart. Only liver and lung membranes showed high specific binding, with dissociation constants (K_d) values of 2.4 ± 0.4 and 0.44 ± 0.05 nM, and maximal binding (B_{max}) of 3.7 ± 0.4 and 1.04 ± 0.01 pmol/mg, respectively. The binding of [G-^{3}H]NBMPR was inhibited in a concentration dependent manner by unlabelled NBMPR, dilazep and a new group of chemically related nucleoside transport inhibitors, mioflazine, soluflazine and R57974, the latter being the most potent derivative. R57974 displaced bound [G-^{3}H]NBMPR as effectively as unlabelled NBMPR suggesting a common binding site. The assay procedure used appears useful for the rapid screening of the effectiveness of nucleoside transport inhibitors which will be of value for the selection of inhibitors suitable for combination with cytotoxic nucleosides in the treatment of selected cancers or parasitic diseases.

The transport of nucleosides across cellular membranes largely represents a carrier-mediated, facilitated diffusion process. This can be inhibited by a number of drugs which either represent nucleoside derivatives including 6-(4-nitrobenzylmercapto)purine ribonucleoside (NBMPR)† and similar nucleoside derivatives or vasodilatory drugs which have no structural similarity to nucleosides, including dipyridamole and dilazep [1–4].

Radiolabelled NBMPR has been used as a ligand presumed to interact directly or closely with the nucleoside transporter in isolated membranes [6–7] and the binding of this ligand appears to correlate well with nucleoside transport inhibition measured in intact cells [8–10]. Thus NBMPR binding could be used to study and identify transporter sites in membranes as well as to measure drug interactions with this system. Using a binding assay with NBMPR as the ligand and isolated membranes is less demanding than actual nucleoside transport assays with isolated membranes and thus is ideally suited for the purpose of drug testing and screening.

A group of chemically related compounds, mioflazine, soluflazine and R57974 [11, 12], have been shown to protect against prolonged normothermic global ischemia in dog hearts. Their cardiovascular effects appeared to be related to their ability to inhibit cellular nucleoside transport [13]. These compounds can be administered orally and thus are of interest regarding potential clinical use for host protection against cytotoxic nucleoside effects in anti-cancer [14] or antiparasitic therapy [15, 16]. Their ability to inhibit nucleoside transport in isolated erythrocytes from different species has been documented [17]. In this paper we report data on the effects of mioflazine and its derivatives on the binding of radiolabelled NBMPR to membrane preparations from different hamster tissues, comparing their effects to those of dilazep and unlabelled NBMPR.

MATERIALS AND METHODS

Materials and drugs. [G-^{3}H]NBMPR (23 Ci/mmol, >98% radiochemically pure) was purchased from Moravek Biochemicals, (Brea, CA). Non-radioactive NBMPR and its 5′-monophosphate derivative (NBMPR-P) were gifts from Prof. A. R. P. Paterson, University of Alberta, Cancer Research Unit. 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 - pyridinyl) - butyl] - *N* - (2,6 - dichlorophenyl) - 1 - piperazineacetamide · 2HCl) and R57974 (2 - (aminocarbonyl) - 4 - [5,5 - bis(4 - fluoro - phenyl)pentyl] - *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 Hofmann-La Roche Co. (Basel, Switzerland).

Membrane preparations. Male hamsters (Syrian-Golden, 150–160 g) were anesthesized with diethyl

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† Abbreviations used: NBMPR, 6-(4-nitrobenzyl)mercaptopurine riboside; NBMPR-P 6-(4-nitrobenzyl)mercaptopurine riboside-5′-monophosphate.

ether, given 0.2 ml heparin (1000 US units/ml i.p.) and the tissues were excised. The tissues were immediately washed in ice-cold 50 mM Tris–HCl buffer (pH 7.4) and homogenized with the same buffer in a Fisher Scientific Stedfast homogenizer Model SL600 at 4°. The homogenized samples were centrifuged twice at 1,000 *g* for 5 min and the supernatants were again centrifuged at 10,000 *g* for 5 min. The membrane pellets resulting from this centrifugation were washed twice and suspended in Tris–HCl buffer at approximately 10.0 to 30.0 mg/ml protein and stored frozen at −20°. Protein concentrations were determined as described by Hartree [18].

Binding assay. Membranes were incubated at concentrations of 2.5–5 mg/ml protein with [G-^{3}H]NBMPR (concentration range 0.3–7.5 mM) in 50 mM Tris–HCl buffer, pH 7.4, in a total volume of 200 μl at 22°. In assays where the effects of drugs (dilazep, mioflazine, soluflazine, R57974) on the binding of [G-^{3}H]NBMPR were determined, the ligand concentration was chosen to be close to the respective K_d (i.e. 0.5 nM for lung membranes and 1 nM for liver membranes). NBMPR-P (sodium salt) was dissolved in water; NBMPR, mioflazine and its derivatives were dissolved in dimethyl sulphoxide at a concentration of 10 mM and rapidly diluted into warmed Tris–HCl buffer to a concentration of 10 μM. Competing drugs were included in assays within the concentration range of 1 nM to 1 mM, and nonspecific binding was determined in the presence of 10 μM unlabelled NBMPR. The incubations were for 30 min and were terminated by adding 1.0 ml ice cold buffer to the assay tubes and immediate centrifugation (14,000 *g*, 1 min) in an Eppendorf 5414 microcentrifuge. The membrane proteins were washed once with ice-cold buffer and dissolved overnight in 0.5 M KOH (100 μl). After addition of 10 ml scintillation cocktail (Optifluor, Amersham), radioactivity associated with the membrane pellets was measured by liquid scintillation counting.

The NBMPR binding parameters (K_d and B_{max}) were determined from Scatchard plots using a computer program by Elsevier-Biosoft ("Dose-effect analysis with microcomputers") performing a straight line fit (B/F vs B) to data sets. The concentration of competing drugs causing 50% inhibition (IC_{50}) of NBMPR binding were interpolated from semi-log plots of drug concentration versus inhibition of specific binding. In these experiments the concentrations of [G-^{3}H]NBMPR was 1 nM and 0.5 nM in the liver membranes and lung membranes, respectively.

Dissociation of bound [G-^{3}H]NBMPR. Membranes were incubated with 1 nM [G-^{3}H]NBMPR under standard conditions described above for 10 min. After centrifugation the pellets were resuspended by vortexing in 100 μl of Tris–HCl buffer containing high concentrations of drugs (NBMPR, dilazep or R57974). At time intervals, the dissociation reaction was terminated in the routine manner, i.e. by adding 1 ml cold buffer followed by immediate centrifugation at 14,000 *g* for 1 min, and processed for radioactivity counting of membrane-associated label.

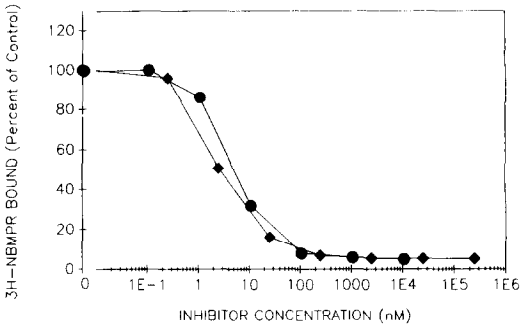

Fig. 1. Effect of NBMPR (–●–) and NBMPR-P (–◆–) as competing ligands on the specific binding of [G-^{3}H]NBMPR to liver membranes. Means of triplicate assays are shown; error bars have been omitted for clarity (SE values being below 6% of the means).

RESULTS

NBMPR binding was assessed in membranes from four tissues, including liver, lung, kidney and heart. In all cases the centrifugation method for separation of bound from free ligand resulted in at least 98% precipitation of protein. Two washes of membranes resulted in stable levels of nonspecific binding and additional washing steps did not reduce this parameter further. Fresh and frozen/thawed membrane preparations produced the same results.

The use of the nucleotide derivative NBMPR-P instead of the nucleoside NBMPR is of general advantage because of the higher solubility of NBMPR-P. Its use is justified only if the system under study contains 5'-nucleotidase or phosphatase activity which rapidly dephosphorylates NBMPR-P to the free nucleoside since only the latter is the active drug or ligand with respect to the nucleoside transporter system [19, 20]. This is to be expected with the membrane preparations used here, and Fig. 1 demonstrates that indeed there is no difference between the effects of NBMPR and NBMPR-P when competing for [G-^{3}H]NBMPR binding in liver membranes. It is reasonable to expect that high levels of phosphorylytic enzymes are also present in the other membrane systems used in this study, thus NBMPR-P was employed as a competing ligand throughout.

Only liver and lung membranes yielded sufficiently high specific binding to permit meaningful data analysis such as by Scatchard plots. In membranes from heart and kidney specific binding only amounted to about 50% or less of total binding at all concentrations of radioactive NBMPR. The resulting binding curves and derived Scatchard plots showed unacceptable variations in data points and therefore further studies were limited to membranes from hamster liver and lung.

Figure 2 shows data on the equilibrium binding of [G-^{3}H]NBMPR to hamster liver and lung membranes. Over 80% of the total binding represented specific binding in both tissues. Data were processed by Scatchard analysis, and the mean estimates (±SE; three experiments) for the dissociation constant K_d and the maximal number of binding sites, B_{max}, were found to be 2.4 ± 0.3 nM and 3.7 ± 0.4 pmol/mg for

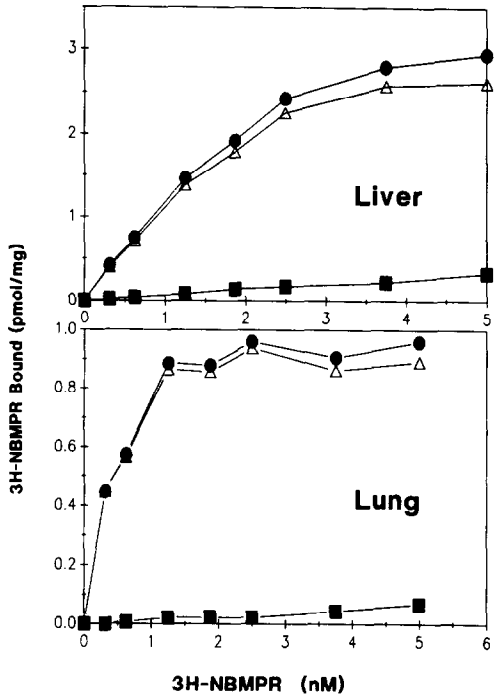

Fig. 2. Concentration dependence of the binding of [G-^{3}H]NBMPR to liver and lung membranes. Total binding (-●-), nonspecific binding (-■-) and specific binding (-△-), calculated as the difference between total and nonspecific binding, are shown. Means of triplicate assays are shown; error bars have been omitted for clarity (SE values being below 3% of the means).

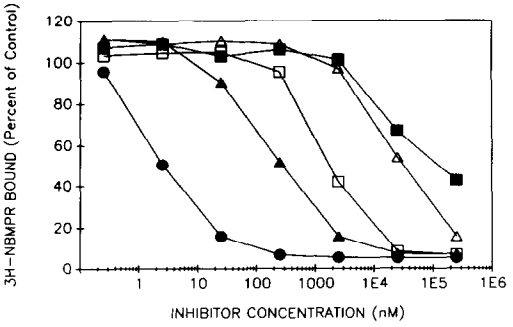

Fig. 3. Effect of NBMPR-P (-●-), dilazep (-▲-), mioflazine (-■-), soluflazine (-△-) and R57974 (-□-) on the specific binding of 1 nM [G-^{3}H]NBMPR to liver membranes. Means of triplicate assays are shown; error bars have been omitted for clarity (SE values being below 5% of the means). The control binding value was 1.07 pmol/mg.

liver membranes, and 0.44 ± 0.05 nM and 1.04 ± 0.01 pmol/mg for lung membranes.

The influence of the non-nucleoside transport inhibitors dilazep, mioflazine, soluflazine and R57974, as well as unlabelled NBMPR, on the specific binding of NBMPR to liver and lung membranes was evaluated in the experiment shown in Fig. 3 All compounds inhibited the specific binding of NBMPR in a concentration dependent manner. The IC_{50} values obtained from this experiment are listed in Table 1. None of the compounds had any significant effect on nonspecific binding.

The rate of dissociation of specifically bound [G-^{3}H]NBMPR from binding sites in liver membranes was assessed in the presence of excess cold ligand and in the presence of high concentrations of dilazep and R57974. These results are shown in Fig. 4. NBMPR-P alone caused about 90% dissociation of bound ligand within 20 min. The effect of R57974, alone or in combination with NBMPR-P, essentially was identical to that of NBMPR-P. However, dilazep when added alone or in combination with NBMPR-P, drastically reduced the rate of dissociation of radioactive NBMPR after an initial rapid phase which lasted for about 5 min and during which time the rate of dissociation appeared similar to that obtained in the presence of the other drugs.

DISCUSSION

The selection of a rather crude membrane preparation and of the centrifugation method for separation of bound from free ligand for this study was purposeful because we wished to explore the suitability of simple, rapid and convenient methodology for the screening of nucleoside transport inhibitors in various tissues. Such studies are of interest in view of the potential clinical relevance of these types of drugs as host protectors against the deleterious actions of cytotoxic nucleosides during anti-cancer or anti-parasitic therapy as demonstrated in animal experimentation [14–16, 21]. We also chose rapid centrifugation as the method for bound/free ligand separation rather than the more elaborate filtration through glassfibre or other membranes.

Our results generally showed that the experimental approach selected appears to be feasible with membrane preparations from hamster lung and liver, but less workable with those from kidney and heart. This may be due to the existence of a very low density of transporter of NBMPR binding sites in some tissues or to the inexplicable presence of a very high nonspecific binding component as seen in our work. Whether or not these observations with specific tissues also apply to other species or are typical of hamster only, cannot be stated at present. This question is under current investigation. It has been shown that there are considerable differences in the capacity of brain membranes from different species to bind NBMPR [6].

Several investigators have addressed the question of whether the binding of NBMPR, a potent nucleoside transport inhibitor, occurs to functional transporter sites, i.e. those understood to bind and transport permeant nucleosides. Binding of NBMPR was expected to indicate the number or density of transporters, and thus the transport capacity and perhaps physiological importance, of a given tissue or membrane system. To date there are no studies which seriously dispute the general concept that indeed *in vitro* NBMPR binding reflects the presence of nucleoside carriers or transporters in membranes [8, 22, 23]. Also, the inhibition of NBMPR binding by drugs generally appears to indicate that such drugs

Table 1. IC_{50} values for the inhibition of NBMPR (1 nM) binding by nucleoside transport inhibitors as obtained from three experiments (c.f. Fig. 3) with hamster membrane preparations. Geometric means and 95% confidence limits are given

Nucleoside transport inhibitor	Membrane source	
	Liver	Lung
NBMPR-P	1.84 (0.95–3.55) nM	0.86 (0.68–1.08) nM
Dilazep	147 (95–229) nM	28.8 (8.9–93) nM
Mioflazine	67.8 (56–82) μM	6.54 (3.7–11.6) μM
Soluflazine	20.9 (14–31) μM	14.5 (9.6–21.9) μM
R57974	1.72 (1.5–2) μM	0.049 (0.023–0.11) μM

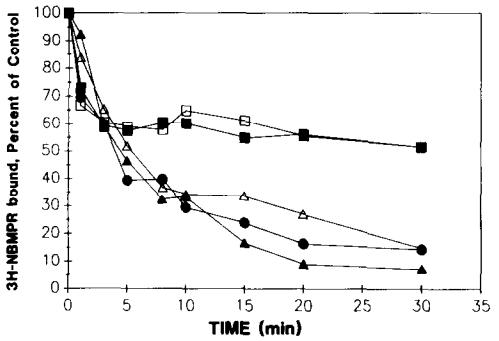

Fig. 4. Rates of displacement of specifically bound [G-^{3}H]NBMPR by different drugs including 20 μM NBMPR-P (-●-), 25 μM dilazep (-□-) and 20 μM R57974 (-△-) as well as combinations of 20 μM NBMPR-P plus 25 μM dilazep (-■-) and 20 μM R57974 (-▲-). Means of triplicate assays are shown; error bars have been omitted for clarity (SE values being below 5% of the means).

are capable of inhibiting nucleoside transporters in intact cells [8, 22, 23]. But clear evidence of whether or not the binding site of NBMPR and that of competing drugs is identical with the binding and carrier site for permeant nucleosides such as adenosine is lacking. A clear understanding of these mechanistic relationships would provide confidence in using such a system for the purpose of *in vitro* screening studies with alternative, non-nucleoside transport inhibitors such as the mioflazine derivatives focussed on in this study.

Mioflazine and its derivatives inhibit adenosine transport in a concentration dependent manner in isolated erythrocytes from different species [17], and their cardiovascular effects have been attributed to their nucleoside transport inhibitory capacity [13]. The present data indicate that these compounds also inhibit *in vitro* NBMPR binding in isolated membranes. Their relative potencies in both membrane preparations were similar, and the shape of the binding competition curves seen in Fig. 3 supports the tentative view that indeed these compounds affect the functional transporters in these membranes and that this competition occurs via a single binding site.

It is evident that both NBMPR and dilazep display a higher potency than the mioflazine derivatives in hamster membrane preparations, and this agrees with unpublished data from this laboratory obtained with isolated erythrocytes from the same species on the basis of adenosine transport measurements. R57974 generally was the more potent among the derivatives tested. Whether *in vivo* the same relative potencies would be obtained cannot be predicted since other pharmacokinetic factors such as protein binding may influence *in vivo* effectiveness. It will be of interest to see whether potency relationships among these drugs, as determined by NBMPR binding assays, show similar variations as was evident from studies with isolated erythrocytes. In the latter case it has been observed that in the mouse the mioflazine derivatives are considerably less potent than NBMPR but that in human erythrocytes they are of similar potency [17].

The study of the mechanism of action of non-nucleoside transport inhibitors has been hampered in part because essential tools such as radiolabelled derivatives are not easily available. Shi and Young [7] have shown that in guinea-pig and rat lung both ^{3}H-labelled dipyridamole binds with the same stoichiometry as NBMPR, but whether or not the same site was involved could not be determined. Further, Koren *et al.* [24] have shown that dilazep reduced the rate of dissociation of labelled NBMPR from its binding site while the permeant uridine increased it. This finding indicated that dilazep acted via a different site which upon occupation affected binding parameters at the NBMPR site. Differences in the susceptibility of NBMPR binding to brain membranes from different species to the competing (dissociating effects) of nucleoside transport inhibitors such as dipyridamole and diazepam have also been reported [6].

We thus investigated whether dissociation of NBMPR from its binding site in hamster liver membranes is also affected by dilazep or R57974, the most potent nucleoside transport inhibitor among the mioflazine derivatives. The data shown in Fig. 4 indicate that R57974 does not interfere with the dissociation of NBMPR, and thus there is no evidence against the concept that mioflazine derivatives bind directly to the NBMPR binding site. However, dilazep caused the same phenomenon described by Koren *et al.* [24], i.e. the dissociaton of NBMPR was strongly reduced in its presence, an effect that occurred after an initial lag phase of about 5 min (Fig. 4) and was also seen when NBMPR-P was present as well. This suggests again that dilazep acts via a site different from that involved in NBMPR

binding, with strong interactions between these sites. These may be allosteric sites on the same protein transporter system or may be different proteins. The involvement of a conformational change in the transporter/NBMPR binding site system with accompanying changes in the rates of dissociation or association of NBMPR, and different affinities of an inhibitor drug to these sites, has been suggested by Hammond and Clanachan [6] in their study on NBMPR binding to cortical membranes.

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