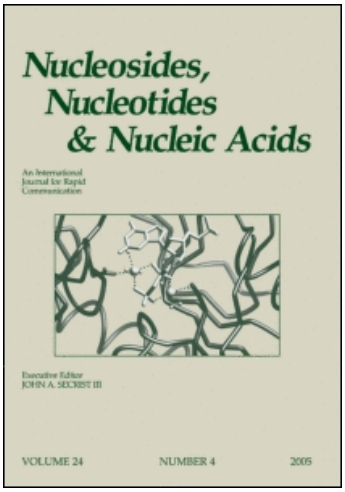

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Comparative Pharmacology of Nucleoside Transport Inhibitors

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COMPARATIVE PHARMACOLOGY OF NUCLEOSIDE TRANSPORT INHIBITORS

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ABSTRACT - The few existing nucleoside transport inhibitors are almost equipotent on the transporter in washed human erythrocytes. Large differences exist however in tightness of binding and activity in the presence of human plasma, as well as in *ex vivo* inhibition and duration of action when given intravenously or orally. Examples are presented of the marked effect of nucleoside transport inhibition on adenosine accumulation in ischemic myocardium. That may explain the remarkable cardioprotection in several models when a nucleoside transport inhibitor with an appropriate pharmacokinetic profile (such as R 75 231) is applied.

INTRODUCTION

Two chemically unrelated drugs, dipyridamole (DIP) and dilazep (DIL) were introduced in the early nineteen sixties as potent and specific nucleoside transport inhibitors, and were followed by hexobendine (a relative of DIL). Some years later we added a third chemical entity, lidoflazine, to the list. More recently we developed newer analogs such as mioflazine (MIOFL) and R 75 231[1] which were more potent transport inhibitors with much less calcium antagonistic properties. Nitrobenzylthioinosine (NBTI) should also be mentioned, although it is mostly used as a biochemical tool to locate and characterize the transporter protein; being a nucleoside analog, it may be of limited therapeutic use in man.

POTENCY OF EXISTING NUCLEOSIDE TRANSPORT INHIBITORS

The human red blood cell is well suited for the evaluation of drugs which interact with the nucleoside transporter; it can be collected easily and quickly, remains viable for a long time, is representative of the human transporter and originates from the eventual therapeutic target species.

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[1] R 75 231: 2-(aminocarbonyl)-N-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis (4-fluorophenyl)-pentyl]-1-piperazineacetamide.

A simple way to explore nucleoside transport is by adding adenosine (ADO) to a dilute suspension of washed human erythrocytes and to follow the decrease in optical density at 265 nm. More information can be gathered if HPLC is applied to estimate not only the disappearance of ADO, but at the same time the extracellular appearance of inosine (INO) and hypoxanthine (the latter being the end product in human red blood cells). One advantage of this procedure is that INO can be added instead of ADO, thus excluding the possible effects of drugs on adenosine deaminase (ADA). As far as the disappearance of ADO is concerned, both the spectrophotometric and the HPLC procedure yield the same IC_{50}-values for the existing drugs. However, as shown in Table 1, the sensitivity of ΔINO is higher, which may be explained by an additional inhibitory effect of the drugs on the rate of efflux of INO, formed inside the erythrocyte. The extrusion of INO under these conditions is remarkable in the light of the general belief that the ADA-activity in human erythrocytes is less than the activity of the purine nucleoside phosphorylase (PNP). Apparently, the intracellular PNP-activity is regulated (by extracellular phosphate?).

Several years ago, we found that some nucleosides, at micromolar concentrations, prevented the continuous release of inorganic phosphate (Pi) derived from 2.3-diphosphoglycerate catabolism in human erythrocytes. The explanation was that intracellular Pi is trapped by these nucleosides in the PNP-reaction so that it was logical to assume that prevention of the transport of the nucleosides would reestablish the rate of Pi-release. This principle provides an easy system for screening a large number of organic molecules for nucleoside transport inhibition (for details see 1). Thus far, we have looked at around 18,000 different structures and none showed any activity at 1×10^{-6} M, in sharp contrast with the marked potency of the reference drugs (Table 2). When comparing the data in Tables 1 and 2, one may notice some discrepancy between the two procedures in that the order of potency is DIL $\eqsim$ NBTI > R 75 231 > MIOFL $\eqsim$ DIP (Table 1), but DIL $\eqsim$ R 75 231 > NBTI $\eqsim$ MIOFL >> DIP (Table 2). We do not have any ready explanation for these findings.

The concentrations of added ADO are sometimes regarded as being unphysiologically high. However, as shown elsewhere (2), similar, or even higher, concentrations may be reached **locally** during ischemia. What really matters, under these conditions, are not the kinetics during the first seconds, but the overall removal during the first minutes of reperfusion. The latter is dependent not only on the transporter, but also on intracellular catabolism, which will dictate the gradient.

From the above experiments one may conclude that DIP, DIL, NBTI, MIOFL and R 75 231 are all potent inhibitors of the nucleoside transporter in washed human erythrocytes, with IC_{50}-values at the 10^{-8} M level. The action is very specific in that we do not know of any other activity at these concentrations. This property is seen only with a very limited number of compounds.

TABLE 1

	ΔADO↓	ΔINO↑	ΔHYP↑
DIL	0.49	0.37	0.69
NBTI	0.53	0.36	0.93
DIP	1.9	1.2	4.8
MIOFL	1.75	1.2	3.6
R 75 231	1.1	0.77	1.45

IC_{50}-values (x 10^{-8} M) of the existing nucleoside transport inhibitors for ΔADO, ΔINO, ΔHYP (HPLC-analysis) in a suspension of human RBC (12.5 µl packed cells in 1 ml buffered saline; 20 min at room temp.; 40 µM ADO added). Average from dose-response curves in 5 donors.

TABLE 2

	INO 10 µM	INO 40 µM	INO40/INO10
DIL	1.5	2.3	1.5
NBTI	3.8	9.5	2.5
DIP	16.0	36.0	2.3
MIOFL	4.0	9.0	2.3
R 75 231	1.5	2.0	1.3

IC_{50}-values (x 10^{-8} M) of the existing nucleoside transport inhibitors as measured by de-inhibition of the release of Pi from human RBC. (12.5 µl packed cells in 1 ml buffered saline; 1 h at 37 °C; INO 10 or 40 µM). Calculated from dose-response curves from 6 different donors.

BIOAVAILABILITY OF EXISTING NUCLEOSIDE TRANSPORT INHIBITORS

While potency is important, the affinity of a drug for its target and the tightness of binding have a major influence on *in vivo* effects. To evaluate this aspect, we first incubated washed human erythrocytes with 1 x 10^{-6} M of these various drugs in buffered saline, and subsequently determined inhibition of nucleoside transport by 1 in 5 dilution in a solution containing INO and then measuring the release of Pi. The original suspension was then quickly centrifuged, the cell pellet taken up in drug-free buffer (10 vols.) and inhibition was

measured again. With DIP, DIL and NBTI, more than 50 % of the inhibitory effect was lost after the first wash and almost all during the next cycle. However, even after 6 washings (theoretically reducing the concentration to below 1×10^{-13}), inhibition by R 75 231 still remained virtually complete.

In another series of experiments, the disappearance of ADO was measured when added to a suspension of erythrocytes in undiluted human plasma. Taking into account breakdown in plasma, which is not affected by these drugs, the IC_{50}-values for DIP and MIOFL were found to be more than 100 times higher than those in washed cells, showing an extensive binding to plasma proteins which thus compete for binding to the transporter. This effect was not seen in dog plasma. The increase in IC_{50}-values, due to binding to human plasma protein, was much less for NBTI (x 20), DIL (x 10) and R 75 231 (x 6). Especially for a drug such as DIP, which is also a potent inhibitor of a specific phosphodiesterase (PDE) isoenzyme in blood vessels (IC_{50}-values $\leq 1 \times 10^{-6}$ M - 3), this protein binding may have important consequences. If the effect on PDE is indeed less affected by protein binding, it may well happen that at the micromolar concentrations required to increase local ADO concentration, cyclic AMP levels are raised due to the action of ADO on specific receptors, and also because of inhibition of cAMP breakdown by PDE. It is possible that this concerted action is the mechanism underlying the so-called "steal" effect of DIP which is seen at very high doses (0.56 mg/kg/i.v.) and which is frequently exploited in thallium imaging for the detection of coronary stenosis. It may not be fair to indict ADO as the only culprit (for references see 4).

The *ex vivo* inhibition of nucleoside transport by drugs injected intravenously can be determined in blood taken at different time intervals. In dogs, no activity could be detected at 0.06 mg/kg of DIP, whereas at 0.13 mg/kg 50 % of inhibition was found for up to 2 hours. The inhibition by DIL (0.07 mg/kg) lasted less than 1 hour (32 % at 30 min). On the contrary, when injecting R 75 231 the *ex vivo* inhibition reached 88 % (1.5 hour) and 46 % (5 h) at 0.05 mg/kg and more than 90 % for up to 5 hours after 0.1 mg/kg. The results of similar experiments in rabbits with doses of 0.1 mg/kg are illustrated in Figure 1. These studies again provided evidence of a very long lasting effect of R 75 231 only.

When given orally to dogs at doses up to 0.3 mg/kg, neither DIP nor DIL showed *ex vivo* inhibition at any time. Oral treatment with R 75 231, 0.06 mg/kg, resulted in 50 % inhibition at 2 hours. At 0.12 mg/kg inhibition was 50 % and 70 % after 2 and 6 hours respectively. These data show excellent oral absorption of R 75 231.

In conclusion, among the few existing nucleoside transport inhibitors, R 75 231 has a unique profile. The binding to the transporter is very strong and activity is barely affected by the presence of plasma proteins. In addition R 75 231 has a long duration of action when given intravenously and is very well absorbed when given orally.

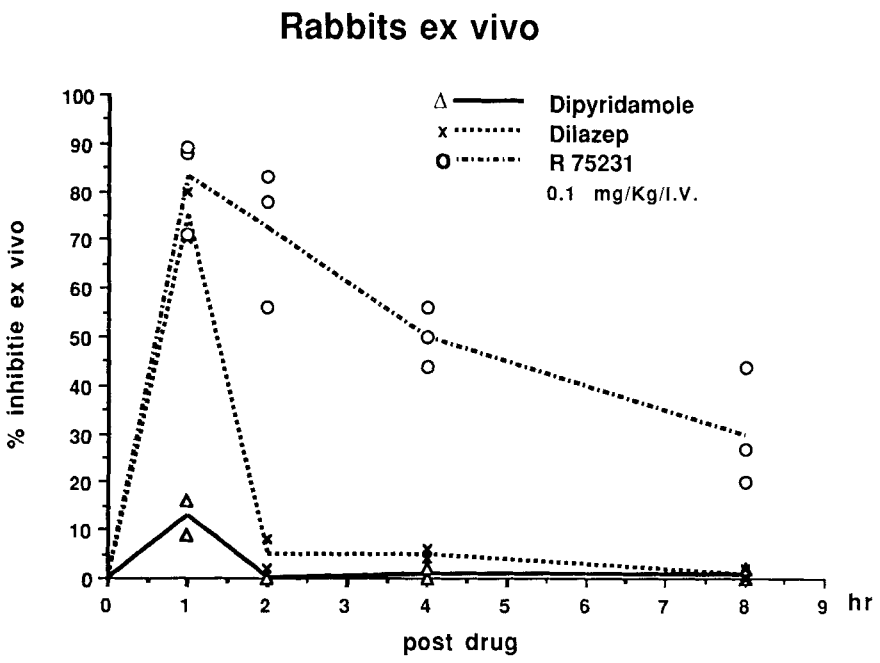

FIG. 1. Ex vivo inhibition of nucleoside transport by dipyridamole, dilazep, and R75231 injected intravenously.

EFFECT OF NUCLEOSIDE TRANSPORT INHIBITION *EX VIVO*

The isolated heart can be regarded as a first approach to the *in vivo* activity of nucleoside transport inhibitors. As discussed elsewhere (5), the transport and catabolism in the endothelial cells lining the coronary capillaries, are major factors in the removal of ADO produced in the interstitial fluid during ischemia. The most remarkable result of nucleoside transport inhibition in rabbit hearts after global ischemia is the inversion of the ratio between ADO and INO in reperfusates. As illustrated in Figure 2, DIL and R 75 231 are very potent in this respect, a 5-fold increase in the ratio being achieved at 2 and 3.2 x 10^{-9} M respectively. DIP was 7 times less potent than DIL and, surprisingly, NBTI was found to be very weak (more than 30 times less potent). Even in hearts isolated from rabbits pretreated with 0.1 mg/kg i.v. of R 75 231 and DIL 2 hours beforehand and perfused with drug-free buffer for 30 min before the induction of ischemia, this effect was still very pronounced. This benefit was not seen with DIP.

We have already reported extensively on the effect of nucleoside transport inhibition on purine metabolism in isolated hearts from cats (2) and guinea pigs (6); also in dog hearts *in*

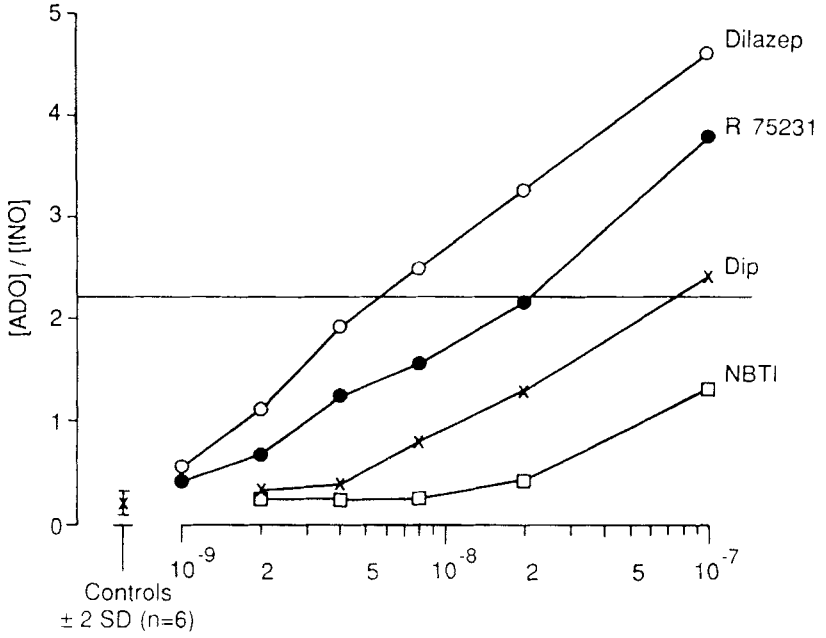

FIG. 2. Effect of increasing doses of different nucleoside transport inhibitors on the ratio between ADO and INO in 2 min reperfusates from rabbit hearts subjected to 14 min of global ischemia.

situ during ischemia (7) and reperfusion (8). All evidence points in the same direction: nucleoside transport inhibition prevents not only the otherwhise rapid catabolism of ADO - presumably by the endothelial cells - but at the same time its escape into circulation. The latter is an the important difference between transport inhibition and inhibition of ADA as seen for instance with deoxycoformycin. The net result is the prolonged presence of ADO in the interstitial space of the ischemic area where it can exert its many beneficial effects.

CARDIOPROTECTION BY NUCLEOSIDE TRANSPORT INHIBITION

As discussed elsewhere (4), ADO has a pharmacological profile which makes it an almost unique substance to handle the many problems arising during ischemia and reperfusion. It is also produced in response to ischemia in heart, brain and kidney. It is therefore not surprising that drugs which prolong the actions of ADO evince remarkable cardioprotection in experimental models. In earlier studies we reported the protection by LIDO and MIOFL in dog hearts *in situ*, subjected to 1 hour of normothermic global ischemia (for references see 9). Thus far, no other classes of drug, including calcium antagonists, beta-blockers, or anything else have shown any effect in this severe model.

Recently, it was found that R 75 231 at 0.1 mg/kg i.v. in pigs significantly prevented death due to ventricular fibrillation during LAD occlusion (from 90 % in controls to 20 % when treated - 10).

Successful (7 out of 7) transplantation has been achieved after preservation of canine donor hearts for 24 hours in a cold cardioplegic solution containing R 75 231 whereas no heart (out of 7) functioned beyond 1 hour in its absence (W. Flameng - in preparation). The accumulation of ADO was very prominent in the treated hearts only during the first hour of reperfusion. In this respect a recent report (11) should be mentioned where it was shown that after 2 hours of coronary artery occlusion in dogs, the infusion of ADO during the first hour of reperfusion resulted in a remarkable improvement of function at 3 hours after relief of the occlusion.

A most striking cardioprotective effect of R 75 231 (0.1 mg/kg/i.v.) was found very recently in a series of experiments on the survival of rabbits after l-epinephrine or norepinephrine application subcutaneously (1 and 2.5 mg/kg respectively). Of the 60 animals serving as controls 25 died while the survivors had increased plasma levels of LDH and/or the myocardial isoenzyme (after 24 hours). Pretreatment and, even more importantly, treatment 1 hour after the challenge resulted in only 1 death (n = 60) and significantly lower plasma LDH than in the controls. We are not aware of any such marked effect of other drugs in this model, which is relevant to human myocardial infarction and cardiac failure.

The difference between other classes of drugs and nucleoside transport inhibitors may well be that other drugs are aimed to act specifically on one aspect in the course of myocardial deterioration. By contrast, prolonging the local accumulation of ADO (which is the only effect thus far known for a drug such as R 75 231) may attenuate most, if not all, the harmful processes. The unique pharmacokinetic profile of R 75 231 may explain why such a remarkable cardioprotection has not been seen with drugs such as DIP or DIL, so long already in use in human therapy.

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