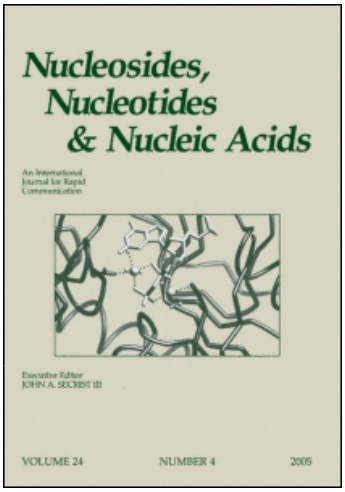

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MODULATION OF [^{3}H]NITROBENZYLTHIOINOSINE BINDING KINETICS

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Abstract: Inhibitors and substrates of the nucleoside transporter were tested for their effects on the kinetics of [^{3}H]nitrobenzylthioinosine binding. Results are discussed in terms of a distinct site mediating the allosteric modulation of [^{3}H]nitrobenzylthioinosine binding affinity.

[^{3}H]Nitrobenzylthioinosine (NBMPR) has gained extensive recognition as a specific probe in studies of ligand interactions with nucleoside transport systems of mammalian cells[1]. Mass law analysis of [^{3}H]NBMPR equilibrium binding data generally yield linear Scatchard plots with Hill coefficients not different from unity, reflecting the existence of a single class of high affinity (K_D < 1 nM) non-cooperative recognition sites. In addition, virtually all compounds that inhibit nucleoside flux also competitively inhibit the binding of [^{3}H]NBMPR. Nevertheless, there are a number of reports describing results with [^{3}H]NBMPR that are not compatible with a simple non-cooperative binding site model: a) [^{3}H]NBMPR site affinity and inhibitor sensitivity vary among species and membrane preparations[1-3]; b) concentration-effect profiles for the inhibition of [^{3}H]NBMPR binding, in some cases, give rise to inhibitor Hill coefficients significantly different from unity[2,4-6], and c) the rate of dissociation of [^{3}H]NBMPR from its sites can be decreased by relatively high concentrations of dipyridamole (DY) and enhanced by nucleosides such as adenosine[1,7-10]. The latter phenomenon has been attributed to either nonspecific membrane perturbations or allosteric binding site interactions, but insufficient data are available to allow for definitive conclusions. This study was undertaken to define more precisely the

pharmacological characteristics of the processes mediating these inhibitor-induced modifications of [^{3}H]NBMPR dissociation kinetics.

Materials and Methods: [^{3}H]NBMPR (16 Ci/mmol) was purchased from Moravek Biochemicals Inc (Brea, Ca). Dilazep was generously provided by Asta Werke, Germany, and mioflazine was a gift from Dr. H. Van Belle, Janssen Research Foundation, Belgium. Diazepam (Hoffmann La Roche, Canada) was obtained from Dr. J.T. Hamilton, University of Western Ontario. CV-1808 (2-phenylaminoadenosine) and cyclopentyladenosine were purchased from Research Biochemicals Inc. All other nucleosides, NBMPR, nitrobenzylthioguanosine (NBTGR), and DY were supplied by SIGMA. Ehrlich ascites cells were grown as an intraperitoneal culture in mice (Swiss, male, ≈ 30 g). Cells were isolated and processed to obtain purified plasma membranes as described previously[4]. Membranes were stored at -80°C in 15% dimethylsulfoxide (DMSO) for up to three months with no loss in [^{3}H]NBMPR binding activity.

Plasma membranes were washed extensively to remove DMSO and incubated with 0.65 nM [^{3}H]NBMPR at 22°C in 50 mM Tris-HCl buffer (pH 7.1) in the presence and absence of competing agents (1 ml final vol). The binding reaction was terminated by vacuum filtration through Whatman GF/B filters followed by two washes with 4 ml of 10 mM Tris-HCl, pH 7.1 at 4°C. For dissociation rate studies, 10 μM NBTGR was included in combination with the test inhibitors to ensure that reassociation was blocked completely. In association rate studies, membranes were not preincubated with inhibitor prior to addition of [^{3}H]NBMPR (preincubation did not affect the results obtained). All values reported were derived from computer-generated best-fit curves (GraphPAD InPlot, v 3.01) to the specific (NBTGR-sensitive) binding data obtained from each experiment.

Results and Discussion: Equilibrium binding studies revealed a single type of [^{3}H]NBMPR recognition site in Ehrlich cell membranes with characteristics compatible with a nucleoside transport inhibitory site (TABLE 1). Kinetic studies yielded [^{3}H]NBMPR association (k_{+1}) and dissociation (k_{-1}) rate constants of 1.22 (± 0.04) X 10^9 $M^{-1}min^{-1}$ and 0.080 ± 0.001 min^{-1} (reassociation blocked with 10 μM NBTGR alone), respectively (n=28). DY inhibited the dissociation of [^{3}H]NBMPR from Ehrlich cell membranes at concentrations which were within the range used

TABLE 1: Effect of nucleoside transport inhibitors and substrates on the kinetics of [^{3}H]NBMPR binding to Ehrlich cell membranes.

	Binding $K_i(\mu M)^A$	Dissociation $EC_{20}(\mu M)^B$	Association $IC_{50}(\mu M)^C$	$t_{\frac{1}{2}}^{max}(min)^C$
Dilazep	0.0015 ± 0.0001	1.1 ± 0.1 (↓)	0.025	5.3
DY	0.060 ± 0.007	2.2 ± 0.1 (↓)	12	43[D]
CV-1808	0.381 ± 0.069	123 ± 7 (↑)	5	5.2
Diazepam	13 ± 1	41 ± 1 (↑)	38	3.1[D]
Adenosine	44 ± 3	786 ± 47 (↑)	192	5.4

[A] Inhibition of the equilibrium binding of [^{3}H]NBMPR (mean ± SEM, n≥5).
[B] Concentration which induces a 20% shift in the dissociation rate (k_{-1}) of [^{3}H]NBMPR (mean ± SEM, n≥5; ↑↓ represent direction of change).
[C] Derived from hyperbolic plots of inhibitor concentration versus half-time for [^{3}H]NBMPR association, as described in the text (n≥3).
[D] Significantly different from adenosine (Students t-test, $P<0.05$).

to inhibit equilibrium binding (10^{-7} - 10^{-4} M). These results may explain the relatively shallow concentration-inhibition curves for DY (Hill coefficients < 0.8) that have been observed in some [^{3}H]NBMPR binding studies[4,5, and unpublished results]. Dilazep and mioflazine were also effective inhibitors of [^{3}H]NBMPR dissociation (IC_{50} values of 3.6 and 3.9 μM, respectively). In contrast, nucleosides enhanced the rate of dissociation of [^{3}H]NBMPR with an order of potency similar to their order of affinity for the transporter (2'-deoxyadenosine > adenosine > thymidine > uridine > 2'-deoxycytidine > cytidine). CV-1808 and cyclopentyladenosine also enhanced the rate of dissociation of [^{3}H]NBMPR with potencies similar to that of adenosine, even though they were 100-times more effective than adenosine as inhibitors of nucleoside flux in Ehrlich cells (data not shown) and [^{3}H]NBMPR binding (TABLE 1). Similar results were obtained using membranes solubilized with octylglucoside, indicating that this phenomenon is not likely due to nonspecific membrane perturbations. Rather, these results are compatible with a model in which the relative capacity of an agent to enhance the rate of dissociation of NBMPR reflects its interaction with the permeant site of the transporter. It is of interest to note that, of the agents tested, diazepam, a benzodiazepine, was the most effective enhancer of [^{3}H]NBMPR dissociation.

Further differences between the various inhibitors were apparent from their influence on the rate of association of [^{3}H]NBMPR (TABLE 1). Plots of inhibitor concentration versus half-time for [^{3}H]NBMPR association ($t_{½}$) described simple hyperbolas with origins equal to the $t_{½}$ in the absence of inhibitor (0.824 min). IC_{50} values for inhibition of [^{3}H]NBMPR association and $t_{½}^{max}$ values (extrapolated maximum $t_{½}$) shown in TABLE 1 were derived from these relationships. DY had the greatest effect on $t_{½}$ (60-fold increase). Indeed, in the presence of 10 μM DY (which inhibits equilibrium binding by 80%), [^{3}H]NBMPR required almost to two hours to reach binding equilibrium. A similar effect of DY on [^{3}H]NBMPR association rate has been described for rat lung membranes[10]. This striking depression of association rate appeared specific for DY, since dilazep, which is similar to DY in its effect on [^{3}H]NBMPR dissociation, induced only a 7-fold increase in association $t_{½}$.

In conclusion, the specific binding of [^{3}H]NBMPR can be modified by ligand interactions with a site distinct from the [^{3}H]NBMPR binding site. These interactions occur at pharmacologically relevant concentrations of inhibitors and should be taken into consideration in the design and analysis of [^{3}H]NBMPR binding studies, particularly where protocols involve concentrations of inhibitors in excess of 1 μM. The relationship of this site to nucleoside transport function awaits further investigation on the molecular structure of the binding proteins involved.

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