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INHIBITION BY NITROBENZYLTHIOINOSINE OF UPTAKE OF ADENOSINE, 2′-DEOXYADENOSINE AND 9-β-D-ARABINOFURANOSYLADENINE BY HUMAN AND MOUSE ERYTHROCYTES*

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Abstract—Nitrobenzylthioinosine was previously shown to inhibit transport of uridine and thymidine across the plasma membrane of the human erythrocyte. This report shows that treatment of human and mouse erythrocytes with nitrobenzylthioinosine, nitrobenzyldeoxythioinosine or hydroxynitrobenzylthioguanosine reduced formation of metabolites from extracellular adenosine, 2′-deoxyadenosine and ara-A, apparently through inhibition of mediated transport of these compounds into the cells. With human erythrocytes, the most potent inhibitor of the three was nitrobenzyldeoxythioinosine.

Transport of uridine and thymidine across the plasma membrane of human erythrocytes is inhibited by nitrobenzylthioinosine† (NBMPR) and various structurally related compounds [1–5]. Inhibition of transport results from binding of NBMPR with high affinity ($K_{dissoc} = 10^{-9}$ M) to sites in the erythrocyte membrane that appear to be part of the uridine–thymidine transport mechanism [6, 7]. The uridine transport mechanism of the human erythrocyte has a broad substrate specificity; uridine exchange diffusion was accelerated by a variety of permeants, including adenosine and arabinosyladenine (ara-A), but not significantly by 2′-or 3′-deoxyadenosine [1–3].

This report describes inhibitory effects of NBMPR and two related compounds on the formation of metabolites from extracellular ara-A, adenosine and 2′-deoxyadenosine by intact mouse and human erythrocytes; the effects are attributed to inhibition of entry of adenine nucleosides into the cells.

MATERIALS AND METHODS

Erythrocytes were obtained by centrifugation (1700 *g*, 15 min) of: (a) human blood after 21–28 days of storage at 4° in Acid Citrate-Dextrose (ACD) solution A (U.S.P.)‡ and (b) heparinized blood from Ha/ICR or BDF_1 mice (Health Sciences Animal Breeding Unit, University of Alberta). Erythrocytes were washed three times (with centrifugation at 1700 *g* for 15 min) in medium A (Krebs–Ringer phosphate solution at pH 7·4 containing 5 mM glucose), or medium B [140 mM NaCl, 1·4 mM $MgSO_4$ and 18 mM *N*-Tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid at pH 7·4] and resuspended in medium A or B. Hematocrits were determined by a capillary method and cell numbers with a model F Coulter Counter.

To determine whether pretreatment with NBMPR and related compounds inhibited transport of labeled adenosine, 2′-deoxyadenosine or ara-A, formation of metabolites of the nucleosides in suspensions of pretreated erythrocytes was compared before and after sonic disruption of the cells. For pretreatment, cells were suspended in 1 vol. of medium A, with and without inhibitor, and were incubated for 15 min at 37°. After centrifuging, cell sediments were then resuspended in 24 vol. of medium A, and portions of each suspension were chilled and treated four times with 15-sec bursts of 20 kc ultrasound with 2 -min cooling periods between each burst.

Formation of metabolites from the labeled nucleoside substrates was assayed in incubation mixtures consisting of equal volumes (usually 1·0 ml) of the cell preparations just described and medium A containing radioactive nucleoside. Portions of incubation mixtures with intact cells were reserved for determination of cell content (hematocrit); the mixtures were then incubated at 37° with gentle shaking and, at timed intervals, 100-μl samples were added to chilled (4°) tubes containing 5 μl of 42% $HClO_4$. After 15 min, these samples were neutralized by addition of 5 μl of a KOH solution exactly equivalent to the $HClO_4$ solution. After centrifuging to remove $KClO_4$, 10-μl portions of the supernatants were chromatographed with appropriate carrier bases, nucleosides and nucleotides using the two-dimensional thin-layer system of Crabtree and Henderson [8]; chromatograms were first developed in acetonitrile–ammonium acetate (0·1 M, pH 7)–ammonia (60:30:10), dried, and developed twice in 1-butanol–methanol–water–ammonia (60:20:20:1). Carrier compounds were located by their fluorescence under u.v. light, and radioactivity was determined by the direct counting of chromatogram sections (1·5 × 2 cm) in a liquid scintillation system. For each experiment, determinations of radioactivity were made under identical conditions to avoid variation in counting efficiencies.

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† 6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine.

‡ Red Cross Blood Transfusion Service, Edmonton, Alberta.

To assay binding of [7″-^{14}C]NBMPR to erythrocytes, washed cells were incubated for 15 min at 25° in 9 vol. of medium B containing graded concentrations of [7″-^{14}C]NBMPR; after centrifuging incubation mixtures (1700 *g*, 15 min), medium samples were reserved for ^{14}C-assay and cells were washed by suspension in 5 vol. of medium B and resedimented (1700 *g*, 15 min). Concentrations of [7″-^{14}C]NBMPR in cell and medium samples were determined from their ^{14}C-content: (a) 0·4-ml portions of the cell sediments were dried at room temperature on absorbant strips* which were then combusted in a Packard model 305 sample oxidizer for assay of ^{14}C [9] and (b) ^{14}C present in medium samples was determined by liquid scintillation counting in Bray's fluor [10].

Nitrobenzyldeoxythioinosine† (dNBMPR) was a gift from Dr. M. J. Robins, Department of Chemistry, University of Alberta, Edmonton, Alberta. NBMPR and hydroxynitrobenzylthioguanosine‡ (HNBTGR) were purchased from Raylo Chemicals Ltd., Edmonton, Alberta. Synthesis of [7″-^{14}C]NBMPR§ ($2{\cdot}01 \times 10^7$ cpm/μmole) was reported earlier [6]. [8-^{14}C]adenosine (58·1 mCi/mmole), [8-^{14}C]-deoxyadenosine (44·7 mCi/mmole) and [2-^{3}H]ara-A (11 Ci/mmole) were commercial products.

RESULTS

To determine whether inhibitors of uridine and thymidine transport [1, 2, 7] also inhibit the uptake of extracellular adenine nucleosides by erythrocytes, the effects of pretreatment of intact erythrocytes with NBMPR on the metabolism of adenine nucleosides was compared before and after sonic disruption.

Human erythrocytes

The data of Table 1 indicate that prior treatment with NBMPR impaired the ability of intact erythrocytes, when incubated in NBMPR-free medium, to convert extracellular adenosine to inosine, hypoxanthine and nucleotides. The NBMPR effect was evidently due to inhibition of adenosine entry into the cells, because: (a) the experiments of Table 2 showed that pretreatment with NBMPR was without effect on the formation of adenosine metabolites by disrupted erythrocytes, and (b) NBMPR is known to inhibit transport of other nucleosides in erythrocytes [1, 2].

Table 1. Inhibition by NBMPR of adenosine uptake*

NBMPR (μM)	Time (min)	Content (nmoles/ml) in total incubation mixture (cells + medium)			
		Adenosine	Inosine	Hypoxanthine	Nucleotides
0	0	161·1	2·9	1·4	0
	10	96·4	15·5	57·2	1·2
	20	37·6	25·7	94·1	1·7
10	0	166·0			
	20	141·5	0·4	13·1	1·5
	40	110·5	5·7	25·5	2·2

* Human erythrocytes were incubated in 1 vol. of medium A with or without NBMPR for 15 min at 37°; after centrifugation, cells were resuspended in 24 vol. of medium A. Portions (1·0 ml) of the cell suspensions were added to 1·0-ml portions of medium A containing [^{14}C-8]-adenosine (final concentration, 81·5 μM) and incubated at 37° for the periods indicated, whereupon mixtures were chilled and treated with perchloric acid. The neutralized "acid-soluble fractions" of each incubation mixture were assayed chromatographically for [8-^{14}C]-adenosine and ^{14}C-metabolites.

Since previous studies [1, 2] had indicated that compounds structurally related to NBMPR inhibited transport of uridine, NBMPR, dNBMPR and HNBTGR were compared for the ability to inhibit formation of metabolites of extracellular adenosine in the experiment of Fig. 1; the most effective inhibitor of the three was dNBMPR. Although pretreatment with 3·5 μM HNBTGR had little effect on adenosine uptake (Fig. 1), pretreatment with higher concentrations was inhibitory (data not shown).

Uptake of 2′-deoxyadenosine and ara-A by intact erythrocytes was also inhibited by prior treatment with NBMPR or HNBTGR (Table 3), whereas metabolite formation was similar in sonicated portions of inhibitor-treated and untreated cells. As with

* "Telfa" pads; Kendall Co. (Canada), Toronto, Ontario.

† 6-[(4-Nitrobenzyl)thio]-9-β-2′-deoxy-D-ribofuranosylpurine.

‡ 2-Amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine.

§ 6-[(4-nitro-[7-^{14}C]-benzyl)thio]-9-β-D-ribofuranosylpurine.

Table 2. Effect of NBMPR on adenosine metabolism by intact and sonicated human erythrocytes*

Initial adenosine concn (μM)	Pretreatment with NBMPR (μM)	Adenosine conversion			
		Rate (nmoles/min/ml cells)		Percentage	
		Intact	Sonicate	Intact	Sonicate
81·5	0	0·205	0·284	100	100
81·5	10·0	0·043	0·280	21	99
309	0	0·293	0·282	100	100
309	10·0	0·055	0·305	19	108

* The procedure described in Table 1 was followed except that, after treatment with and without NBMPR, portions of the cell suspensions were sonicated. [8-^{14}C]Adenosine and its ^{14}C-metabolites (data not shown) were determined by chromatography and the rates of adenosine disappearance were determined as in Table 1.

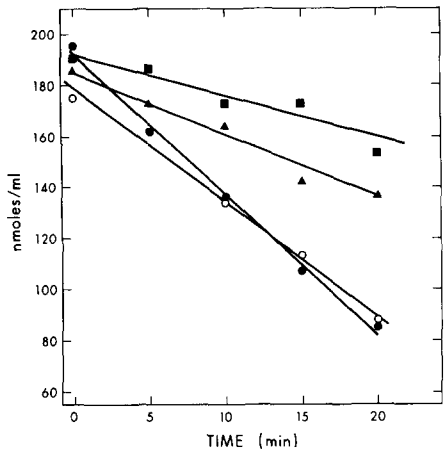

Fig. 1. Inhibition of adenosine uptake by human erythrocytes. Cells were pretreated by incubation for 15 min at 37° in medium A containing 3·5 μM NBMPR (▲), 3·5 μM dNBMPR (■), 3·5 μM HNBTGR (○) or no additive (control) (●). Cells were collected by centrifugation and resuspended in 24 vol. of medium A; 1·0-ml portions of these suspensions were mixed with 1·0-ml medium A containing [8-^{14}C]adenosine (final concentrations were about 190 μM), and incubated at 37°. Incubation mixture samples (cells plus medium) were assayed chromatographically for adenosine (presented here) and its metabolites (not shown).

adenosine (Fig. 1), transport of 2′-deoxyadenosine was more sensitive to inhibition by dNBMPR than by the other inhibitors. When the preincubation medium contained both NBMPR and dNBMPR, each at 1·75 μM, the apparent inhibition of transport of 2′-deoxyadenosine and that of adenosine was not significantly greater than that observed for each with 3·5 μM dNBMPR (data not shown).

Mouse erythrocytes

Pretreatment with NBMPR inhibited uptake of ara-A by intact mouse erythrocytes, since conversion of ^{3}H-ara-A to its metabolites was reduced by intact cells, but was unaffected in sonicates of the same cells (Fig. 2). In the experiment of Fig. 2A, NBMPR pretreatment had these effects: (a) the rate (μmoles/min/ml of cells) of ara-A catabolism was reduced from

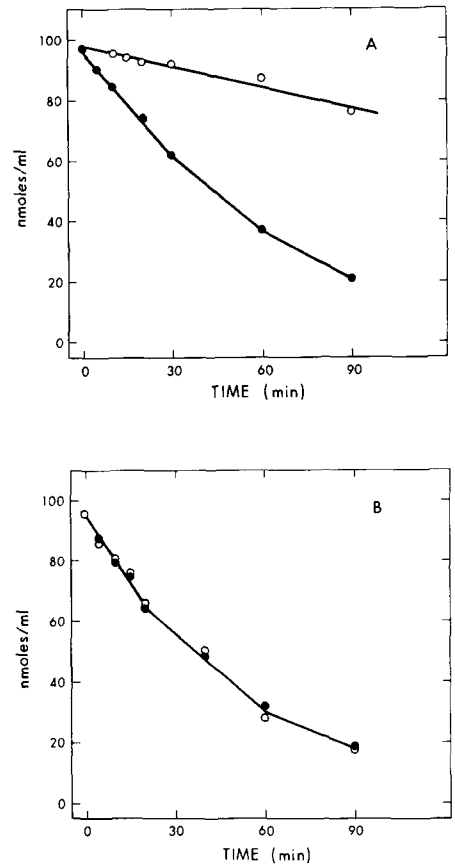

Fig. 2. Effect of NBMPR pretreatment on uptake of ara-A by mouse erythrocytes. Erythrocytes from BDF$_1$ mice were incubated in medium A with (○) or without (●) 10 μM NBMPR for 15 min at 37°. Cells were collected by centrifugation, resuspended in medium A and portions of the suspensions were sonicated. Portions (1·0 ml) of the various cell preparations were incubated with 1·0-ml portions of medium A containing [2-^{3}H]ara-A (final concentrations were about 100 μM). Samples from preparations containing intact cells (panel A) and cell sonicates (panel B) were extracted as described in the text and were assayed chromatographically for [2-^{3}H]ara-A (presented here) and its metabolites (not shown).

Table 3. Effect of nucleoside transport inhibitors on the metabolism of 2′-deoxyadenosine and arabinosyladenine by human erythrocytes*

Nucleoside and initial conc (μM)	Pretreatment		Nucleoside utilization			
			Rate (μmoles/min/ml cells)		Percentage	
	Inhibitor	(μM)	Intact	Sonicate	Intact	Sonicate
2′-deoxyadenosine 103	None		0·134	0·156	100	100
103	NBMPR	10	0·086	0·184	64	118
90	None		0·193	0·199	100	100
90	HNBTGR	10	0·145	0·216	75	109
196	None		0·285		100	
196	HNBTGR	3·5	0·258		91	
196	NBMPR	3·5	0·194		68	
196	dNBMPR	3·5	0·116		41	
ara-A 101	None		0·092	0·059	100	100
101	HNBTGR	10	0·053	0·065	58	110

* Experiments were conducted as described in Table 2.

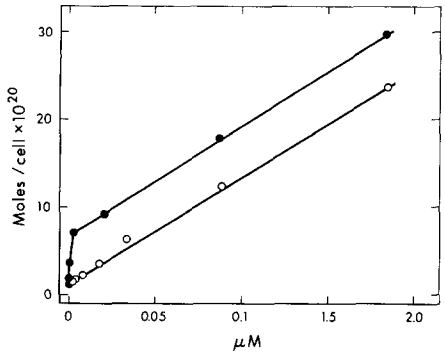

Fig. 3. Binding of NBMPR to mouse erythrocytes. Washed erythrocytes from Ha/ICR mice were incubated for 15 min at 25° in medium B containing [7″-^{14}C]NBMPR at the indicated concentrations in the absence (●) or presence (○) of 50 μM HNBTGR. Incubation mixtures were centrifuged to obtain: (a) medium which was assayed for ^{14}C to determine NBMPR concentrations, and (b) cells which were washed once in medium B, enumerated and assayed for ^{14}C by combustion as described in Materials and Methods.

0·061 to 0·012, and (b) the rate of production of arabinosylhypoxanthine (ara-H), the major metabolite, was reduced from 0·057 to 0·004 (data not shown).

Other experiments with mouse erythrocytes showed that: (a) uptake of 0·1 mM extracellular adenosine by a 2% suspension of intact erythrocytes was inhibited 94% by pretreatment with 10 μM NBMPR, and (b) uptake of 1·2 mM extracellular adenosine by a 10% suspension was inhibited 64% by 5 μM HNBTGR.

Previous reports have shown that NBMPR inhibited transport of uridine and thymidine in human erythrocytes by binding with high affinity (K_{dissoc} = 10^{-9} M) to transport-specific sites in the erythrocyte membrane [7]. The binding of [7″-^{14}C]NBMPR to mouse erythrocytes in the presence and absence of HNBTGR is illustrated in Fig. 3. As with human erythrocytes [7], two modes of retention of NBMPR by mouse erythrocytes were apparent: (a) a component which was proportional to the concentration of NBMPR, and (b) a saturable component which was completely eliminated in the presence of HNBTGR. The latter component appears to be analogous to the membrane-bound component of NBMPR uptake in human erythrocytes, which was displacable by the competing ligands HNBTGR and methylthioinosine [7]. An apparent K_{dissoc} of 4×10^{-9} M for the saturable binding of NBMPR to mouse erythrocytes was obtained from a plot of the reciprocals of saturably bound NBMPR (moles/cell) and the concentration of free NBMPR [6], using data from the experiment of Fig. 3.

DISCUSSION

Studies with intact human erythrocytes and erythrocyte ghosts indicated that nitrobenzylthioinosine binds with high affinity to a particular set of membrane sites [7]. The amount of this "saturably bound" NBMPR was directly related to the degree of inhibition of uridine transport in intact erythrocytes, suggesting that the NBMPR binding sites are part of the uridine transport mechanism.

Specificity of the uridine transport mechanism in human erythrocytes was investigated by an "accelerative exchange diffusion" assay which measured the ability of exogenous nucleosides to accelerate efflux of uridine from erythrocytes preloaded with uridine [1–3]. Uridine efflux was accelerated by adenosine and ara-A, but not significantly by 2′-deoxyadenosine, suggesting that adenosine and ara-A were substrates for the uridine transport mechanism, whereas 2′-deoxyadenosine was not.

Studies of nucleoside transport in other cell types have indicated tissue or species differences in transporter-permeant specificities. Rabbit polymorphonuclear leukocytes appear to have a single nucleoside transport mechanism which accepts pyrimidine and purine nucleosides, including adenosine (K_m = 10 μM), 2′-deoxyadenosine and ara-A [11]. Cultured Novikoff hepatoma cells appear to have an adenosine-specific mechanism (K_m = 6–10 μM) which does not accept pyrimidine nucleosides or other purine nucleosides as permeants [12]. Adenosine transport in dog myocardium occurs by a mechanism that has an affinity for adenosine (K_m = 11·6 μM) which is higher than that for 2′-deoxyadenosine and ara-A [13].

The results presented here indicate that pretreatment with NBMPR and dNBMPR significantly inhibited entry of adenosine, 2′-deoxyadenosine and ara-A. HNBTGR, which was a potent inhibitor of uridine and thymidine transport in erythrocytes [2, 7], was less effective than either NBMPR or dNBMPR. NBMPR and dNBMPR did not show specificity of inhibition toward the transport of either adenosine or 2′-deoxyadenosine, and the failure of these inhibitors to act additively suggests that they interact with the same transport component(s) in the erythrocyte membrane. These results, together with the earlier observation that 2′-deoxyadenosine did not significantly accelerate uridine efflux [2, 3] suggest the existence in the human erythrocyte of an additional nucleoside transport mechanism with permeant and inhibitor specificities which overlap with those of the uridine–thymidine transport mechanism.

The data presented here indicate that inhibitors of nucleoside transport such as NBMPR significantly reduce deamination of ara-A by intact human and mouse erythrocytes through their effects on cellular uptake of ara-A. Ara-A has antitumor activity in some experimental systems [14, 15] and is currently in clinical trial [16,–18]. The usefulness of ara-A as a chemotherapeutic agent is limited by rapid conversion to ara-H, which is therapeutically active and rapidly excreted [15, 19]. Erythrocytes of mice and humans have high levels of adenosine deaminase and constitute a major site of ara-A inactivation [20], suggesting that agents which reduce erythrocyte inactivation of ara-A such as NBMPR might be expected to improve ara-A therapy.

Note added in proof. Since this paper was submitted, Agarwal and Parks [1] have reported the inhibition by nitrobenzylthioguanosine by intact erythrocytes of humans.

REFERENCES

1. C. E. Cass and A. R. P. Paterson, *J. Biol. Chem.* **247**, 3314 (1972).

2. C. E. Cass and A. R. P. Paterson, *Biochem. biophys. Acta* **291,** 734 (1973).
3. J. M. Oliver and A. R. P. Paterson, *Can. J. Biochem.* **49,** 262 (1971).
4. A. R. P. Paterson and A. I. Simpson, *Can. J. Biochem.* **44,** 1423 (1966).
5. A. R. P. Paterson and J. M. Oliver, *Can. J. Biochem.* **49,** 271 (1971).
6. M. A. Pickard, R. R. Brown, B. Paul and A. R. P. Paterson, *Can. J. Biochem.* **51,** 666 (1973).
7. C. E. Cass, L. A. Gaudette and A. R. P. Paterson, *Biochim. biophys. Acta* **345,** 1 (1974).
8. G. W. Crabtree and J. F. Henderson, *Cancer Res.* **31,** 985 (1971).
9. Packard Instrument Co. Inc., *Instruction Manual 2118* for model 305 Sample Oxidizer (1972).
10. G. A. Bray, *Analyt. Biochem.* **1,** 279 (1960).
11. R. A. Taube and R. D. Berlin, *Biochim. biophys. Acta* **233,** 6 (1972).
12. P. G. W. Plagemann, *Biochim. biophys. Acta* **233,** 688 (1971).
13. R. A. Olsson, M. K. Gentry and J. A. Snow, *Biochim. biophys. Acta* **311,** 242 (1973).
14. J. J. Brink and G. A. LePage, *Cancer Res.* **24,** 312 (1964).
15. J. J. Brink and G. A. LePage, *Cancer Res.* **24,** 1042 (1964).
16. G. A. LePage, A. Khaliq and J. A. Gottlieb, *Drug Metab. Dispos.* **1,** 756 (1973).
17. L. T. Ch'ien, J. W. Benton, R. A. Buchanan and C. A. Alford, *Pediat. Res.* **6,** 385 (1972).
18. G. P. Bodey, J. Gottlieb, K. B. McCredie and E. J. Freireich, *Proc. Am. Ass. Cancer Res.* **15,** 129 (1974).
19. G. A. LePage, *Can. J. Biochem.* **48,** 75 (1970).
20. R. Koshiura and G. A. LePage, *Cancer Res.* **28,** 1014 (1968).
21. R. P. Agarwal and R. E. Parks Jr., *Biochem. Pharmac.*, **24,** 547 (1975).