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Inhibition of Uridine Uptake in HeLa Cells by Nitrobenzylthioinosine and Related Compounds

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SUMMARY

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Rates of uridine uptake by logarithmically proliferating monolayer cultures of HeLa cells at 20° were constant for intervals of several minutes, and rate saturability was evident. Nitrobenzylthioinosine (NBMPR), a potent inhibitor of nucleoside transport, abolished saturable uptake of uridine, revealing a nonsaturable component of uptake that apparently was due to simple diffusion. Under conditions of the uptake assay, the principal metabolite of uridine was UTP. NBMPR had no effect on uridine kinase in HeLa cell extracts, or on the relative proportions of uridine anabolites in intact cells. These results indicate that NBMPR inhibited mediated passage of uridine into the cell. The inhibition of uridine transport by NBMPR was characterized by unchanged V_{max} values, with increases in apparent K_m values, whether the inhibitor and permeant were added simultaneously in the uptake assay or the cells had previously been treated with inhibitor. To assess the structural requirements for inhibitory activity, compounds related structurally to NBMPR were tested for their ability to inhibit uridine uptake; of these, the most effective were the 2'-deoxyribosyl and arabinosyl analogues of NBMPR and the S^6-nitrobenzyl derivatives of 6-thioguanosine, 2'-deoxy-6-thioguanosine, and 6-selenoguanosine.

INTRODUCTION

The transport of uridine across the plasma membrane of human erythrocytes has been clearly established as a facilitated diffusion process; these cells do not metabolize uridine (1, 2). In cells that metabolize nucleosides, the transport of such permeants has been studied mainly through initial rate kinetics of uptake.[1] Rate saturability, competition between permeants, and the existence of specific inhibitors have been interpreted to mean that a rate-limiting step in the uptake process is mediated. It is frequently assumed that this step is transport (3, 4). Although difficult to demonstrate rigorously, it has been argued that transport is rate-limiting in uridine uptake by cells that metabolize this nucleoside (5-8). However, the functional independence of transport and subsequent metabolic steps

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[1] Transport refers to the mediated passage of permeant across the plasma membrane; uptake of permeant, which refers to intracellular accumulation of permeant and its metabolites, includes a transport step.

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in the uptake of uridine or other nucleosides has not been established.

A variety of substances chemically unrelated to nucleosides inhibit the transport of uridine; for example, Persantine (5), cytochalasin B (9), and phloretin (10) are inhibitory, changing the kinetics of uptake (V_{max} unaltered, K_m increased), possibly by direct interaction with the transport elements of the membrane or by changing their milieu. Various S^6 derivatives of 6-thio-9-β-D-ribofuranosylpurine (e.g., NBMPR[2]) are potent inhibitors of nucleoside transport. These compounds are evidently specific toward nucleoside permeation because (a) they block cellular uptake of nucleosides, but not of bases, glucose, or amino acids (11), and (b) their aglycones are much less inhibitory (12, 13).

In the present study, a rapid sampling procedure was used to examine the kinetics of uridine uptake and metabolism in HeLa cell monolayers, and inhibition of uridine uptake by NBMPR was compared with that by a number of structurally related compounds.

METHODS

HeLa cell monolayers were maintained in MEM-M (Eagle's minimal essential medium with 10% calf serum) with subculture at weekly intervals. Trypsinized monolayer cultures provided inocula for suspension cultures in MEM-S [MEM without calcium salts and supplemented with 5% calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM HEPES buffer, pH 7.4]. Suspension cultures were initiated in spinner flasks and, after expansion into 2- or 5-liter round-bottomed flasks, were kept under continuous agitation with a Vibro-Mixer (model E1, Chemapec, Hoboken, N. J.) and maintained in exponential growth by dilution. Vibro-Mixer cultures, being single-cell suspensions, were used to prepare replicate monolayer cultures. Cell culture materials were purchased from Grand Island Biological Company. Stock cultures, tested at regular intervals by Dr. Janet Robertson, Department of Medical Bacteriology, University of Alberta, were mycoplasma-free.

Uptake experiments employed replicate monolayer cultures, prepared in 2-ounce prescription bottles (Brockway Glass Company, Brockway, Pa.). Bottles were "conditioned" for 24 hr at 37° with MEM-M (which was then discarded) and then inoculated with 10^6 cells in 4.0 ml of MEM-A (equal volumes of MEM-M and MEM-S). Plating efficiencies were 50–60%, and, after a 4-hr lag, cells proliferated exponentially with doubling times of about 20 hr. The replicate cultures were used 24 hr after inoculation, at which time proliferation was exponential.

Uridine uptake by the replicate monolayer cultures was assayed at 20° as follows; exceptions to this procedure are noted. Each assay was performed in triplicate, and each bottle was passed individually through the following sequence. (a) Growth medium was aspirated, the bottle was then placed horizontally with the cell sheet uppermost, and 4.0 ml of transport medium (MEM-T[3]) containing [5-^{3}H]uridine were added to the bottle. (b) To initiate the uptake interval, the cell sheet was immersed by rapidly rotating the bottle 180° around its long axis. (c) Aspiration of the permeant-containing medium was started 5 sec before the interval ended. (d) To end the uptake interval, the cell sheet was flooded with 60 ml of ice-cold 0.154 M NaCl, which was removed rapidly by aspiration after 15 sec. The bottles were thoroughly drained by a brief centrifugation in the inverted position. Cell sheets were dissolved in 1.5 ml of NCS tissue solubilizer (Amersham/Searle), and the digests were assayed for radioactivity by liquid scintillation counting, using Bray's scintillation fluid (14). Four to eight cultures from each set of replicates were treated with trypsin for determination of cell number with an electronic particle counter. Permeant uptake during assay intervals of 0 sec was estimated by flooding cell sheets with 60

[2] The abbreviations used are: NBMPR, nitrobenzylthioinosine (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; MEM, Eagle's minimal essential medium; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

[3] MEM-T consisted of MEM without serum and bicarbonate, but containing 20 mM HEPES (pH 7.4 at 20°).

ml of ice-cold 0.154 M NaCl containing 0.35 ml of permeant-containing MEM-T [the amount remaining after step (c) above]. Permeant specific activities were determined in the presence of blank NCS cell digests.

Time courses of uridine uptake were linear over extended intervals at 37° and 20°, as demonstrated in Figs. 1 and 3, and extrapolated through zero time. Except as noted, uridine uptake rates were determined as the difference between the cellular content of uridine after 60-sec and 0-sec exposures to medium containing [5-^{3}H]uridine. Rates of nucleoside uptake (picomoles per 10^6 cells per minute) were found to be independent of cell number in the range employed, 0.8–1.5 × 10^6 cells/monolayer.

To examine uridine metabolism, monolayer cultures were exposed to MEM-T containing 4 μM [5-^{3}H]uridine for intervals that were initiated and terminated as in the uptake assay; cultures were processed individually in sets of five in the following manner. After removal of the stopping solution, the first bottle was immediately placed on ice, and at once the cell sheet was extracted for 2 min with 1.5 ml of cold 0.5 M perchloric acid; this extract was passed in succession over four additional cell sheets, each exposed to [5-^{3}H]uridine in the same manner as the first. The extract, then representing five cultures, was neutralized on ice with KOH, using bromcresol purple as an internal indicator. Such extracts were freeze-dried and reconstituted in 300 μl of 1.0 mM phosphate buffer (pH 6.5), and 30-μl portions, along with appropriate carriers (50 nmoles each), were chromatographed on thin layers of cellulose or polyethylenimine cellulose. After development, carrier areas were scraped onto filter paper and combusted in a Packard model 305 Sample Oxidizer for assay of radioactivity by liquid scintillation counting. Chromatographic system I employed polyethylenimine cellulose sheets (MN 300, Machery-Nagel and Company, Brinkmann Instruments) prepared by washing with 4 M sodium formate buffer (pH 3.4) for 18 hr, followed by two 1-hr immersions in 50% methanol. In system I, chromatograms were developed with 0.5 M, 2.0 M, and 4.0 M sodium formate buffers (pH 3.4) employed in succession, without drying between transfers; transfers were made when fronts moved 2.5 and 10 cm above the origins, and development in 4.0 M sodium formate was stopped when the front had moved 17 cm. Chromatographic system II employed Eastman 6064 cellulose thin-layer sheets developed in ethyl acetate–isopropyl alcohol–water (65:22.5:12.5, v/v/v).

Cell extracts, prepared in the following way, were assayed for uridine kinase activity (15). Cell suspensions, about 10^7 washed HeLa cells in 1.2 ml of 0.15 M KCl containing 3 mM mercaptoethanol and 10 mM Tris-HCl (pH 8.5), were disrupted with 20-kc ultrasound and centrifuged at 10,000 × g for 10 min at 4°; the supernatants were assayed for protein content (16). Kinase assay mixtures contained 9 mM ATP, 9 mM $MgCl_2$, 7.5 mM 3-phosphoglycerate, 50 mM Tris-HCl (pH 8.5), 100 μl of cell extract (approximately 600 μg of protein), and 10 μM [2-^{14}C]uridine in a total volume of 400 μl. Assay mixtures were incubated at 37°, and at 5-min intervals 20-μl portions were applied to 2.5-cm^2 squares of DEAE paper, which, after washing with 10 mM sodium phosphate (pH 6.8), were combusted for assay of ^{14}C.

NBMPR and the S^6-(4-nitrobenzyl) derivatives of 6-mercaptopurine, 6-thioguanine, 6-thioguanosine, and the α and β anomers of 2′-deoxy-6-thioguanosine (provided by Dr. G. A. LePage) were prepared by established methods (17). 4-[(4-Nitrobenzyl)thio]uridine was prepared by the method of Sato and Kanaoka (18). [2-^{14}C]Uridine and [5-^{3}H]uridine were commercial products; radiochemical purities, determined by paper chromatography, exceeded 90%.

RESULTS AND DISCUSSION

The effect of relatively high concentrations of NBMPR on uridine uptake by HeLa cells is shown in Fig. 1. When NBMPR was present during the uptake assay, almost complete inhibition of uridine uptake resulted. When NBMPR-treated cells were washed and assayed for

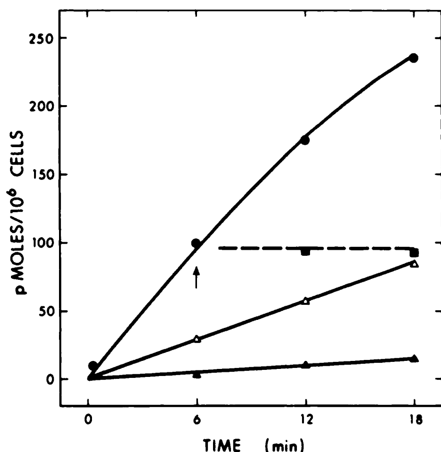

FIG. 1. *NBMPR inhibition of uridine uptake by HeLa cells*

The effect of NBMPR on uridine uptake by replicate monolayer cultures was measured with NBMPR and uridine added together (■, ▲) or after NBMPR treatment (△). In the latter procedure, monolayers were individually incubated at 37° for 6 min in MEM-M containing 56 μM NBMPR and washed in NBMPR-free medium. In the uptake assay, monolayers were incubated at 37° in MEM-M containing 0.7 μM [2-^{14}C]uridine with (▲) or without (●, △) 56 μM NBMPR, or with the latter added (arrow) at 6 min (■).

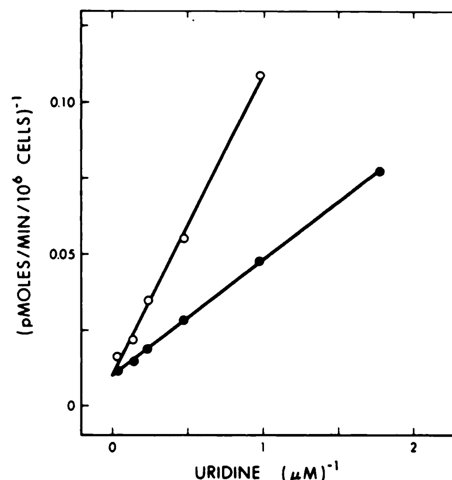

FIG. 2. *Inhibition of uridine uptake in HeLa cell monolayers by prior treatment with NBMPR*

Cultures from a replicate set were individually incubated at 37° for 6 min in MEM-M with (○) or without (●) 62 μM NBMPR and, after washing with MEM-M, were assayed for [2-^{14}C]uridine uptake at 37°, as in Fig. 1; uptake rates were calculated from differences between uptake after 6 and 18 min of incubation. K_m values for control and NBMPR-treated cells were 3.9 and 10.5 μM, respectively, and the common V_{max} value was 95 pmoles/10^6 cells/min.

uridine uptake in NBMPR-free medium, substantial inhibition of this process remained. This persistent inhibitory effect was further examined in the experiment of Fig. 2, which showed that in cells treated with NBMPR and then washed, the V_{max} of uridine uptake was unchanged but the half-saturation constant (K_m) for this process was increased. Evidently NBMPR remained associated with cells during washing and the relatively long intervals of the uridine uptake assays. These results are consistent with reports of the tight, but reversible, binding of NBMPR to erythrocytes (19, 20) and HeLa cells (21).

The kinetics of uridine uptake at 20° was studied with the rapid sampling technique described in METHODS, which provided the time courses of Fig. 3. Because such time courses were linear, cellular uptake of uridine during the first 60 sec measured uptake rate. The data of Fig. 4 show that NBMPR is a potent inhibitor of uridine

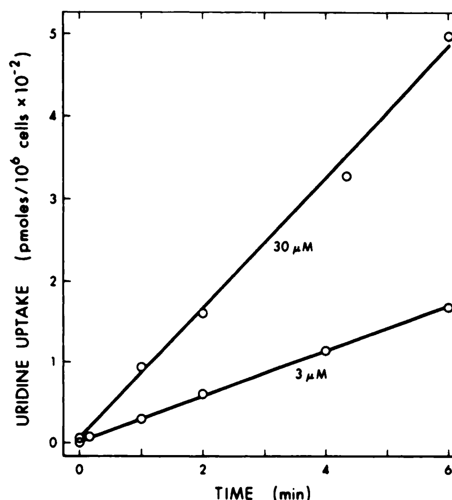

FIG. 3. *Constant rate of uridine uptake by HeLa cell monolayers*

Replicate cultures were incubated at 20° in MEM-T containing 3 or 30 μM [5-^{3}H]uridine, and the cellular content of [5-^{3}H]uridine was determined.

uptake. When cells were incubated in medium containing 3 μM uridine and graded concentrations of NBMPR, uridine uptake rates were reduced by 50% when the NBMPR concentration (IC_{50}) was 0.05 μM; in four experiments, including that of Fig. 4, IC_{50} values ranged between 0.05 and 0.08 μM (mean, 0.06 μM). Figure 4 also demonstrates that prior treatment with NBMPR was somewhat more inhibitory toward uridine uptake than the same concentration of NBMPR added together with [5-^{3}H]uridine in the uridine uptake assay.

The experiments of Fig. 5 showed that the mediated uptake of uridine by HeLa cells was abolished by the presence of 5 μM NBMPR. The rate of uridine uptake was directly proportional to concentration in the medium when the latter contained 5 μM NBMPR, suggesting that this compo-

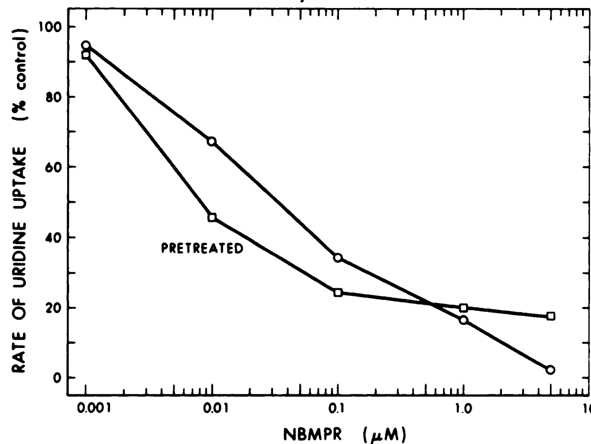

Fig. 4. *NBMPR inhibition of uridine uptake by HeLa cell monolayers at 20°*

The effects of graded concentrations of NBMPR on rates of uridine uptake were determined when 3 μM [5-^{3}H]uridine was added to the replicate cultures together with NBMPR (○) or following treatment with NBMPR (□). In the latter procedure, cell sheets were exposed for 1.0 min to MEM-A containing NBMPR at the indicated concentrations, washed for 30 sec with 4.0 ml of MEM-A, and immediately assayed for uridine uptake. All cultures were processed individually through these procedures. Uptake rates were corrected for diffusional entry of uridine (see Fig. 5 and the text).

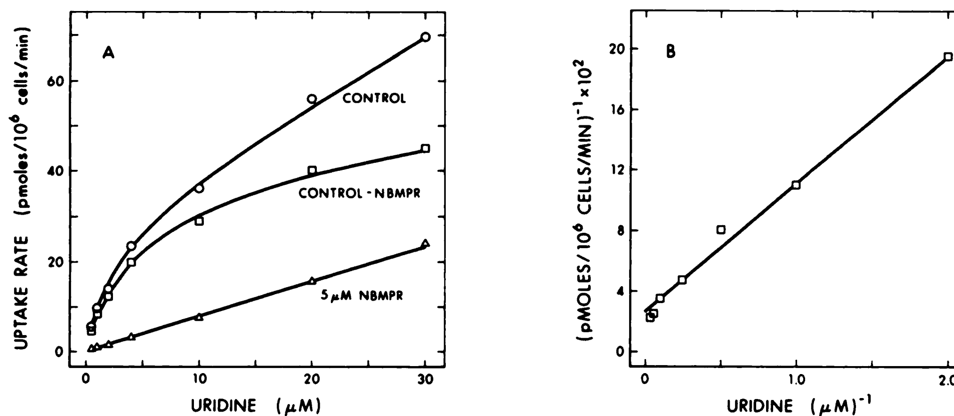

Fig. 5. *Apparent diffusion component of uridine uptake*

Rates of uptake [5-^{3}H]uridine at 20° were determined in the absence (○) and presence (△) of 5 μM NBMPR. A. Uridine uptake rates in the presence of 5 μM NBMPR were attributed to simple diffusion and subtracted from control rates to determine mediated uptake (□). B. Reciprocals of mediated uptake data (□) from A: V_{max}, 39 pmoles/10^6 cells/min; K_m, 2.0 μM.

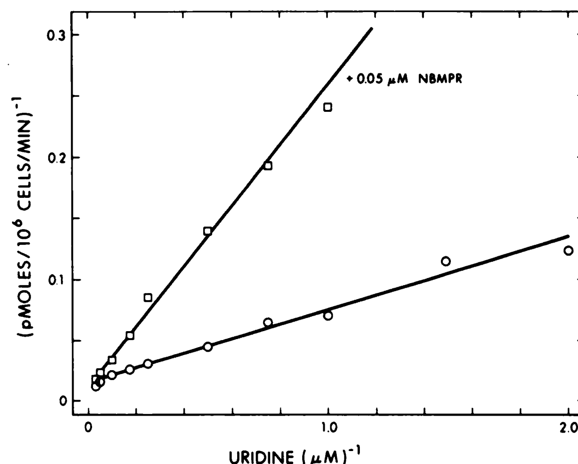

FIG. 6. *Effect of NBMPR on uridine uptake kinetics*

Rates of [5-³H]uridine uptake by replicate monolayer cultures at 20° were measured in the absence (○) and presence (□) of 0.05 μM NBMPR. Rates were corrected for uridine uptake by diffusion, as in Fig. 5. Kinetic constants in the absence of NBMPR: V_{max}, 67 pmoles/10^6 cells/min; K_m, 4.2 μM.

TABLE 1

Failure of NBMPR to inhibit uridine kinase activity

Extracts of sonically disrupted HeLa cells were assayed for uridine kinase activity in the presence and absence of NBMPR.

NBMPR	Uridine kinase[a]
μM	*pmoles/min/mg protein*
0	1.31
2	1.43
10	1.32
25	1.31

[a] Assayed by measurement of uridine phosphorylation.

TABLE 2

Time course of uridine metabolite formation by HeLa cell monolayers

Replicate monolayer cultures were exposed at 20° to MEM-T containing 4 μM [5-³H]uridine; intervals of exposure were ended exactly as in the determination of uptake rate (Fig. 3). The cold saline stopping solution was removed after 15 sec, monolayers were extracted immediately with cold perchloric acid, and the extracts were analyzed by chromatography. ³H accompanying the chromatographic carriers listed accounted for more than 95% of that applied to the chromatograms. Radioactivity present in cytidine and its phosphates was insignificant.

NBMPR	Incubation interval	Uridine	UMP	UDP	UTP
	sec	*cpm/30 μl extract*			
−	10	1,046	104	119	2,019
	30	648	312	389	5,459
	60	896	486	717	9,658
	90	783	723	993	13,035
+	10	687	36	14	274
	60	695	83	63	913

nent of uridine uptake was due to simple diffusion. It is also possible that this component of uridine uptake could be due to a NBMPR-insensitive nucleoside uptake process with a very low affinity for uridine. When gross uptake rates were corrected for the apparent diffusion component (Fig. 5A), the saturability of uridine uptake rates was evident (Fig. 5B). The following values for rates of apparent uridine diffusion at 20° were determined in separate experiments: 0.8 (Fig. 5), 1.1, and 0.9 pmoles/10^6 cells/min/unit of uridine concentration (1.0 μM); the mean (0.9) was applied where indicated as a correction to gross rates of uridine uptake.

The influence of concentration on uridine uptake rates in the presence and absence of 0.05 μM NBMPR was evaluated in the experiments of Fig. 6; V_{max} for uridine uptake was essentially unchanged in the inhibited cultures, but the apparent K_m value was increased. Similar results (inhibition without change in V_{max}) were ob-

TABLE 3

Inhibition of uridine uptake

Rates of uptake of [5-^{3}H]uridine (3 or 4 μM) by HeLa cell monolayers were measured at 20° in the absence and presence of at least three concentrations of each test compound. Control uptake rates of 3 and 4 μM uridine were 19.7 ± 3.6 and 32.0 ± 3.8 pmoles/min/10^6 cells (mean ± SD for 11 and six determinations, respectively). IC_{50} values [concentrations of test compound which reduced uridine uptake rates to 50% of the uninhibited (control) rate] were estimated from plots of inhibition percentage against log inhibitor concentration; concentration ranges of the test compound included the IC_{50} value (Table 3A). Partially inhibitory compounds (Table 3B) are listed together with the highest concentration tested and the inhibition percentage obtained at that concentration. Compounds with little or no effect on uridine uptake at the concentration indicated are listed in Table 3C. Compounds were obtained from the following sources: A, commercial; B, this laboratory; C, M. J. Robins, University of Alberta; D, L. B. Townsend, University of Utah; E, J. P. Miller and R. A. Long, ICN Nucleic Acid Research Institute, Irvine, CA; F, Drug Research and Development, National Cancer Institute, Bethesda, Md.; G, K. C. Tsou, University of Pennsylvania; H, H. J. Schaeffer, Medical College of Virginia.

A. Inhibitory compounds

No.	Test compound	Source	IC_{50}
			μM
914	2-Amino-6-[(4-nitrobenzyl)thio]-9-β-D-2′-deoxyribofuranosylpurine	B	0.03
947	6-[(4-Nitrobenzyl)thio]-9-β-D-arabinofuranosylpurine	E	0.04
554	6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine	B	0.06
844	6-[(4-Nitrobenzyl)thio]-9-β-D-2′-deoxyribofuranosylpurine	C	0.10
218	2-Amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine	B	0.1
852	2-Amino-6-[(4-nitrobenzyl)seleno]-9-β-D-ribofuranosylpurine	D	0.1
861	6-[(4-Nitrobenzyl)thio]-9-β-D-2′-*O*-methylribofuranosylpurine	C	0.1
120	6-(Benzylthio)-9-β-D-ribofuranosylpurine	F	0.25
938	6-(Benzylthio)-9-β-D-arabinofuranosylpurine	E	0.5
555	2-Amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine	B	1.0
915	2-Amino-6-[(4-nitrobenzyl)thio]-9-α-D-2′-deoxyribofuranosylpurine	B	1.0
862	6-[(4-Nitrobenzyl)thio]-9-β-D-3′-*O*-methylribofuranosylpurine	C	2.4
937	6-*N*-(3-Methylbut-2-enylamino)-9-β-D-arabinofuranosylpurine	E	4.0
925	6-[(4-Nitrophenacyl)thio]-9-β-D-ribofuranosylpurine	B	4.5
946	6-(Allylthio)-9-β-D-arabinofuranosylpurine	E	5
926	6-(Benzylthio)-9-β-D-ribofuranosylpurine cyclic 3′,5′-monophosphate	E	10
944	6-(Ethylthio)-9-β-D-arabinofuranosylpurine	E	10
943	6-(Thiocyanato)-9-β-D-arabinofuranosylpurine	E	40
867	4-[(4-Nitrobenzyl)thio]-1-β-D-ribofuranosylpyrimid-2-one	B	40
942	6-(Methylthio)-9-β-D-arabinofuranosylpurine	E	70

served with cells that had received prior treatment with NBMPR. However, analogy with enzyme kinetics, which would suggest competition between permeant and inhibitor, is evidently not valid, because inhibition of nucleoside uptake (a) by a variety of substances structurally unrelated to nucleosides or (b) by prior treatment of cells with *p*-chloromercuribenzoate (22) or with NBMPR (as reported here) is also characterized by unchanged V_{max} values.

Since the principal fate of uridine in HeLa cells is conversion to uridine phosphates (see below), it was possible that NBMPR inhibition of uridine uptake resulted from inhibition of uridine phosphorylation. The data of Table 1 show that NBMPR did not inhibit uridine kinase activity in cell extracts. Table 2 indicates that, while NBMPR significantly reduced the incorporation of external uridine into cellular uridine nucleotides under conditions of the uptake assay, the relative distribution of [5-^{3}H]uridine anabolites among UMP, UDP, and UTP was the same in the presence and absence of NBMPR. Thus NBMPR did not appear to inhibit the intracellular anabolism of uridine, and inhibition of uptake at the level of permeation was indicated. Although most of the radioactivity recovered was in

TABLE 3—*Continued*

B. Partially inhibitory compounds

No.	Test compound	Source	Concentration	Inhibition
			μM	%
109	6-(Methylthio)-9-β-D-ribofuranosylpurine	A	10	29
940	6-(3-Pyridylmethylthio)-9-β-D-arabinofuranosylpurine	E	10	45
928	6-(Methylthio)-9-β-D-ribofuranosylpurine cyclic 3′,5′-monophosphate	E	10	31
948	6-Thio-9-β-D-arabinofuranosylpurine	E	100	25
945	6-Sulfenamide-9-β-D-arabinofuranosylpurine	E	100	17
939	6-Hydrazino-9-β-D-arabinofuranosylpurine	E	100	31
941	6-(β-Hydroxyethylamino)-9-β-D-arabinofuranosylpurine	E	100	35
	Colchicine	A	200	38

C. Compounds with little or no effect

No.	Test compound	Source	Concentration
			μM
924	6-[(4-Nitrobenzyl)thio]purine	B	1
923	2-Amino-6-[(4-nitrobenzyl)thio]purine	B	1
920	2-Amino-6-thio-9-β-D-2′-deoxyribofuranosylpurine	A	1
863	1-N^6-Etheno-9-β-D-ribofuranosylpurine	G	1
922	6-Amino-9-β-D-2′-*O*-methanesulfonylribofuranosylpurine	C	10
921	2-Amino-9-β-D-2′,3′-anhydroribofuranosylpurine	C	10
866	1-N^6-Etheno-2-aza-9-β-D-ribofuranosyladenine	G	10
927	6-(Ethylthio)-9-β-D-ribofuranosylpurine cyclic 3′,5′-monophosphate	E	10
929	6-Thio-9-β-D-ribofuranosylpurine cyclic 3′,5′-monophosphate	E	10
931	N^6-Ethyl-9-β-D-ribofuranosyladenine cyclic 3′,5′-monophosphate	E	10
933	N^6-Hydroxy-9-β-D-ribofuranosyladenine cyclic 3′,5′-monophosphate	E	10
934	N^6-Benzyloxy-9-β-D-ribofuranosyladenine cyclic 3′,5′-monophosphate	E	10
935	N^6-Benzyl-9-β-D-ribofuranosyladenine cyclic 3′,5′-monophosphate	E	10
930	N^6-Methyl-9-β-D-ribofuranosyladenine cyclic 3′,5′-monophosphate	E	10
	2-Amino-6-thiopurine	A	10
	Showdomycin	A	10
918	2′-Deoxycoformycin	F	187
868	9-*erythro*-(2-Hydroxy-3-nonyl)adenine hydrochloride	H	187

UTP, the principal metabolite of uridine, free uridine was present in perchloric acid extracts of cells incubated in the presence and absence of NBMPR (Table 2); similar results were obtained when the experiment of Table 2 was repeated, except that a somewhat greater proportion of radioactivity was present in extracts as free uridine.

The uridine uptake mechanism of HeLa cells appeared to be specific for uridine, since in the presence of 10 μM thymidine [for which the K_m for uptake by these cells is about 0.5 μM (23)], the kinetics of uridine uptake was unaffected and essentially the same as that of the controls in Fig. 6. In similar experiments, the presence of uridine had little effect on rates of cellular uptake of thymidine (23), adenosine (24), or guanosine.[4]

Human erythrocytes transport uridine by a facilitiated diffusion mechanism, which functions independently of uridine metabolism. NBMPR inhibits this transport process by binding with high affinity to specific receptor sites in the erythrocyte plasma membrane (19, 20). In HeLa cells, the lack of a NBMPR effect on uridine metabolism, together with the capacity of NBMPR to eliminate the saturable uptake of uridine (as shown with erythrocytes), suggests that the inhibition of uridine uptake by NBMPR occurs by interference

[4] A. R. P. Paterson and C. E. Cass, unpublished observations.

with the transport portion of the uptake process; a similar conclusion has been reported for NBMPR inhibition of thymidine uptake in HeLa cells (23).

Table 3 summarizes experiments which tested compounds related to NBMPR for their ability to inhibit uridine uptake by HeLa cells. These results demonstrate that (a) the pentosyl and 6-(4-nitrobenzyl)thio groups contribute importantly to the inhibitory activity, (b) the 2′-hydroxyl group of NBMPR does not appear to be involved in the inhibitor-transporter interaction and an *O*-methyl group is accommodated at this position, (c) the presence of an amino group at the purine 2-position does not affect the inhibitor-transporter interaction, (d) 3′-*O* substituents are less well tolerated than those at the 2′-*O* position, (e) NBMPR and its seleno homologue have comparable inhibitory activity in this system, (f) of the two possible configurations for the N^9-glycosidic bond, the β anomer is preferred, and (g) the cyclic 3′,5′-monophosphate derivatives are not accepted by the inhibitor receptor site as readily as the corresponding free nucleosides. 4-(Nitrobenzylthio)uridine (compound 867) was far less inhibitory than its purine homologue, NBMPR, suggesting that the inhibitor receptor site is purine-specific.

NBMPR did not inhibit the uptake of uracil or the following other low molecular weight compounds, indicating a specific inhibitory effect against uptake of nucleosides. Uptake experiments were performed as described for uridine, in the presence and absence of 25 μM NBMPR, using the following permeants (data not shown): [5-^{3}H]uracil (1, 5, and 25 μM), D-[*U*-^{14}C]glucose (1, 5, and 10 mM), L-[4,5-^{3}H]leucine (0.05, 0.5, and 5 mM), and a mixture of 15 L-[*G*-^{3}H]amino acids[5] (0.1, 1, and 10 μM).

In a previously reported study, various nucleoside derivatives, some related to NBMPR, were compared for their ability to inhibit a transport-dependent aspect of nucleoside metabolism in human erythrocytes (25). 6-Benzylthio, 6-benzylamino, and 6-benzyloxy derivatives of 9-β-D-ribofuranosylpurine displayed similar inhibitory potency, and the hydrophobicity of the 6-position substituents appeared to contribute importantly to inhibitory activity, suggesting strong interaction with a hydrophobic region of the inhibitor receptor site. A number of compounds are potent inhibitors in both the erythrocyte and HeLa cell assay systems, and while there may be differences between the two cell systems in the binding affinities of one inhibitor relative to another, there does not appear to be distinct cell-inhibitor specificity.

Kinetic studies, including those reported here, suggest that human erythrocytes (1, 2, 26) and HeLa cells (23, 24) possess nucleoside uptake mechanisms which differ in important respects. Nucleosides are transported in erythrocytes by a mechanism[6] with low specificity toward nucleoside permeants and low affinity for the latter relative to other cells. By contrast, in HeLa cells, four mechanisms of nucleoside uptake[7] are distinguishable by kinetic criteria and by changes in activity during the replication cycle.[8] It is not known whether these mechanisms are independent or whether the specificities evident are imparted at the levels of transport or metabolism in the uptake process. Despite these functional dissimilarities, the structure-activity data reported here for NBMPR inhibition of nucleoside transport, when compared with data reported elsewhere for erythrocytes (20, 25, 26), in-

[5] The mixture consisted of equal concentrations of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

[6] A criterion for permeant specificity in the erythrocyte system was the acceleration of exchange diffusion of [^{14}C]uridine induced by structurally related test permeants; such an assay would not identify additional mechanisms with overlapping specificities.

[7] The following uptake mechanisms are distinguishable: (a) uridine (this work), (b) thymidine and certain thymidine analogues (23), (c) adenosine (24), and (d) inosine and guanosine.[4]

[8] C. E. Cass and A. R. P. Paterson, unpublished observations.

dicate a strong resemblance between the NBMPR receptor sites of erythrocytes and HeLa cells.

Note added in proof: Eilam and Cabantchik (27, 28) have reported recently that nitrobenzylthioinosine is a very potent inhibitor of uridine uptake by MCT cells, a cultured line of hamster cells, and have suggested that binding of the inhibitor may induce a conformational change in the transporter which reduces affinity of the latter for uridine.

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