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Inhibition of nucleoside uptake in HeLa cells by nitrobenzylthioinosinate*

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Transport-specific elements of the plasma membrane mediate the passage of nucleosides into animal cells [1–3]. Various S^6-derivatives of 6-thiopurine nucleosides are potent inhibitors of nucleoside transport; these inhibitions are specific in that uptake of sugars, amino acids and nucleobases is not inhibited. Nitrobenzylthioinosine, the best known member of this group, binds tightly, but reversibly, to specific sites in the plasma membrane; occupancy of these sites in human erythrocytes [4] and HeLa cells [5] results in inhibition of nucleoside transport. In this report, we describe the synthesis of the 5′-monophosphate derivative of nitrobenzylthioinosine (nitrobenzylthioinosinate) and the inhibition by this compound of nucleoside uptake in HeLa cells.

Chemical synthesis. Thioinosinate (6-thio-9-β-D-ribofuranosylpurine 5′-phosphate) was prepared from 6-thioinosine (10 m-moles) by reaction with 3 ml phosphorus oxychloride and 30 ml trimethyl phosphate at 0° [6]. After 2 hr, the reaction was terminated by the addition of 200 ml of cold anhydrous diethyl ether, followed by vigorous stirring for 5 min; after allowing the syrup to settle, the ether phase was discarded. Cold triethylammonium bicarbonate (1 M, pH 7.5) was immediately added to the syrup until the pH of the resulting solution was 7.0. After 2 hr at 0°, the reaction mixture was dried under vacuum at 30° and co-evaporated three times with water, slurried with 10 g silicic acid (60–200 mesh) in water and finally dried under vacuum. The dry product was then added to the top of a dry-packed silicic acid column (3 × 30 cm) and eluted with the following solvents: 800 ml acetonitrile followed by 500 ml each of 18, 22, 25 and 30% 1 N ammonium hydroxide in acetonitrile. Eluate fractions (10 ml) containing thioinosinate [identified by thin-layer chromatography (t.l.c.) on cellulose in isobutyric acid–NH_4OH[conc]–water (66:1:33, v/v)] were freeze-dried and the product so obtained (yield 72 per cent) was judged to be authentic on the basis of the u.v. absorption spectrum [7], chromatographic behavior and susceptibility to hydrolysis by snake venom 5′-nucleotidase (Sigma Chemical Co., St. Louis, MO).

Thioinosinate (0.43 m-mole) was added to a solution of α-bromo-*p*-nitrotoluene (0.50 m-mole) in 2.4 ml of 2.3 M NH_4OH, 8.0 ml of 1,4-dioxane and 4.0 ml tetrahydrofuran. After 1 hr at 20°, the reaction mixture was dried under vacuum at 30°, adsorbed onto 10 g of silicic acid and added to a silicic acid column (2 × 40 cm) as above. The column was eluted with 400 ml acetonitrile followed by 100 ml each of 18, 20, 22 and 25% 1 N NH_4OH in acetonitrile. Eluate fractions (10 ml) containing nitrobenzylthioinosinate [recognized by t.l.c. on cellulose in 95% ethanol–1 M ammonium acetate (7:3, v/v)] were pooled and freeze-dried. The product (yield, 65 per cent) had the following characteristics: u.v. (H_2O): max 287 nm (ε,23,117), min 240 nm (ε 3,986); n.m.r. (Me_2SO-d_6): 4.75 to 3.88 (m, s, 2′, 3′, 4′, 5′ sugar protons), 4.75 (s, 2, benzylic protons), 6.01 (d, 1, J = 6 Hz, $C_1'H$), 7.72 and 8.13 [d (pair), 4 phenyl protons], 8.82 and 8.74 ppm [s (pair), 2, C_2H and C_8H]. Treatment with 5′-nucleotidase converted the product to nitrobenzylthioinosine. Analysis (Microanalytical Laboratory, University of Alberta, Edmonton, Alberta, and Schwarzkopf Microanalytical Laboratories, Woodside, NY) of the monoammonium salt of $C_{17}H_{21}N_6O_9PS \cdot 3\,H_2O$ as calculated was: N, 14.7; P, 5.44; S, 5.61; and as found: N, 14.27; P, 4.96; S, 5.75.

Nucleoside uptake. Replicate monolayer cultures of HeLa S3 cells were prepared as previously described [8];

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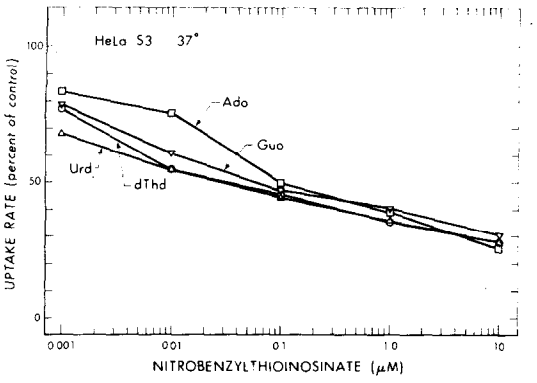

Fig. 1. Inhibition by nitrobenzylthioinosinate of nucleoside uptake by HeLa cells at 37°. Replicate monolayer cultures were exposed for 60 sec to medium containing [^{3}H]nucleoside (at concentrations given in Table 1) and the indicated concentration of nitrobenzylthioinosinate. Rates of nucleoside uptake are expressed as percentages of the following control rates (pmoles/10^6 cells/min) obtained in the absence of inhibitor: adenosine (97.9), guanosine (25.5), thymidine (13.2) and uridine (86.3).

such cultures were in 2-oz prescription bottles and when used contained about 10^6 exponentially proliferating cells with a population doubling time of approximately 20 hr. Measurement of initial rates of uptake of [^{3}H]nucleosides (Amersham/Searle, Oakville, Ontario) with such replicate cultures has been described elsewhere [8, 9]. The cultures (three/condition) were processed individually at 21° and 37° as follows. Growth medium was removed by suction, and the uptake interval (60 sec) was initiated by rapid immersion of cells in [^{3}H]permeant-containing medium [Eagle's minimal essential medium without bicarbonate and supplemented with 20 mM HEPES* (pH 7.4) and 12 mM NaCl]. Five sec before the end of each uptake interval, the cell sheet was flooded with 60 ml of ice-cold 0.154 M NaCl (saline). When uptake at 37° was measured, uptake intervals were ended with 37° saline containing 5 μM nitrobenzylthioinosine. The stopping solutions were removed by rapid suction after 30 sec, and bottles were drained thoroughly. Monolayers were then dissolved in 2.0 ml of 0.5 M KOH and, after mixing with 15 ml of a detergent-xylene fluor solution [10], the resulting solutions were assayed for ^{3}H by liquid scintillation counting.

Figure 1 illustrates inhibition by nitrobenzylthioinosinate of uptake at 37° of adenosine, guanosine, thymidine and uridine by HeLa cell monolayers. The concentrations of inhibitor that reduced uptake to 50 per cent of control values (IC_{50}), derived from the data of Fig. 1 and from similar experiments at 21°, are shown in Table 1. These IC_{50} values are similar to those obtained for nitrobenzylthioinosine inhibition of thymidine [8], uridine [9], adenosine [11], and guanosine† uptake by HeLa cells, indicating that the 5'-phosphorylated derivative is as potent an inhibitor as nitrobenzylthioinosine. These results suggest that nitrobenzylthioinosinate interacts with and is bound, presumably, to the nitrobenzylthioinosine binding site. The chemical identity of the cell-associated inhibitor of nucleoside uptake (nitrobenzylthioinosinate or a product thereof) remains to be determined; it is possible that nitrobenzylthioinosine is the ultimate inhibitor.

Table 1. Inhibition of nucleoside uptake in HeLa cells by nitrobenzylthioinosinate*

[^{3}H]permeant	Concn (μM)	IC_{50} (μM) 37°	IC_{50} (μM) 21°
Uridine	4.0	0.03	0.09
Adenosine	1.0	0.09	0.02
Guanosine	1.0	0.06	0.08
Thymidine	0.5	0.03	0.04

* Replicate monolayer cultures were exposed at 37 and 21° to medium containing the indicated concentration of [^{3}H]nucleoside and graded concentrations of nitrobenzylthioinosinate. The IC_{50} values (concentration of nitrobenzylthioinosinate at which the nucleoside uptake rate was 50 per cent of the uninhibited rate) were obtained from the log dose–response plots of Fig. 1 and from similar experiments at 21°. Control rates (pmoles/10^6 cells/min) of nucleoside uptake at 21°, obtained in the absence of inhibitor, were as follows: uridine (37.4), adenosine (50.7), guanosine (10.5) and thymidine (6.6). Control rates of nucleoside uptake at 37° are given in Fig. 1.

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* *N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

† A. R. P. Paterson and C. E. Cass, unpublished results.