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Differential uptake of [^{3}H]guanosine by nucleoside transporter subtypes in Ehrlich ascites tumour cells

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Intracellular metabolism of [^{3}H]guanosine was minimal (< 15%) during the first 22 s of incubation, and hence reasonable estimates of initial-rate influx kinetics could be derived by using metabolically active cells. Na^+-dependent concentrative [^{3}H]guanosine uptake was not observed. Data suggest that [^{3}H]guanosine was accumulated primarily via the nitrobenzylthioguanosine (NBTGR)-sensitive subtype of facilitated nucleoside transporter. Incubation of cells with 100 nM-NBTGR significantly decreased the potency of guanosine as an inhibitor of [^{3}H]uridine influx. The $V_{max.}$ for [^{3}H]guanosine influx (9.2 pmol/s per μl) was significantly lower than that for [^{3}H]uridine influx (16 pmol/s per μl). The K_m for transporter-mediated [^{3}H]guanosine influx determined in the presence of 100 nM-NBTGR was 16-fold higher (1780 μM) than that determined in its absence, whereas the K_m for [^{3}H]uridine influx was shifted by only 2-fold. In other respects, the cellular accumulations of [^{3}H]guanosine and [^{3}H]uridine were similar; both had K_m values of approx. 140 μM for total mediated influx, and both were inhibited similarly by other nucleosides and transport inhibitors. These characteristics, and the fact that guanosine is an endogenous nucleoside, suggest that [^{3}H]guanosine may prove useful as a poorly metabolized, relatively selective, substrate for study of the NBTGR-sensitive nucleoside transport systems of mammalian cells.

INTRODUCTION

Mammalian cells possess a variety of mechanisms for accumulating nucleosides from the extracellular milieu, including non-concentrative facilitated-diffusion mechanisms [1–3] and concentrative Na^+-dependent transporters [4–7]. The facilitated-diffusion systems can be sub-classified according to their sensitivities to inhibition by S^6-thiopurines, such as nitrobenzylthioinosine or nitrobenzylthioguanosine (NBTGR) [2,3,8–10], and dipyridamole [10–13]. Dipyridamole-sensitivity is largely species-dependent, with nucleoside transporters in the rat being the least sensitive. On the other hand, many cells, including Ehrlich cells [14], express both the NBTGR-sensitive and NBTGR-resistant subtypes of equilibrative transporters. The Na^+-dependent nucleoside transporters can also be sub-classified, according to substrate specificity [3,5]. Given this heterogeneity, and the fact that most nucleosides can serve as substrates for more than one of these systems, it is often difficult to assess confidently the characteristics of a single transporter subtype without considerable pharmacological or biochemical manipulation (e.g. ATP depletion, Na^+ depletion). Another problem is the intracellular metabolism of the transported nucleosides to their nucleotide derivatives. This process leads to an apparent cellular accumulation of [^{3}H]nucleoside against a concentration gradient and confounds the interpretation of experimental data. By convention, 'transport' denotes the transfer of unmodified nucleoside across the cell membrane via a selective, saturable, carrier; 'uptake' refers to the total intracellular accumulation of radioactivity, regardless of the route of entry and metabolic conversions [1–3]. Therefore, in order to measure accurately the membrane transport of nucleosides, it is necessary either to deplete cellular ATP pools [15,16] or to use a non-metabolizable nucleoside analogue such as [^{3}H]formycin B as the transporter substrate [17]. Formycin B, however, is still a substrate for both the NBTGR-sensitive and -resistant equilibrative transporters and at least one of the Na^+-dependent transporters. Ideally, to study selectively the characteristics of one type of transporter in a heterogeneous system one requires a non-metabolized substrate which is selective for the transporter of interest.

In a previous study [16], it was observed that incubation of ATP-depleted Ehrlich cells with 50 nM-NBTGR, to block the NBTGR-sensitive transporter, decreased the ability of guanosine to inhibit 10 μM-[^{3}H]uridine influx, but had minimal effects on the inhibitory potencies of other nucleoside substrates. These results suggested that guanosine was a relatively poor substrate for the NBTGR-resistant transporters in Ehrlich cells. The present study was therefore undertaken to assess the potential of [^{3}H]guanosine to serve as an experimental substrate for studies focused on the NBTGR-sensitive equilibrative transporters of Ehrlich cells.

EXPERIMENTAL

Materials

The Ehrlich ascites tumour cell line was established at the University of Western Ontario from cells provided by Dr. R. M. Johnstone, Department of Biochemistry, McGill University, Montreal, Canada. [5,6-^{3}H]Uridine (35–50 Ci/mmol) and [8-^{3}H]guanosine (3.5–7.0 Ci/mmol) were purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.) and Sigma Radiochemicals (St. Louis, MO, U.S.A.) respectively. ^3H_2O (1 mCi/g) and [*carboxyl*-^{14}C]-dextran-carboxyl (0.58 mCi/g) were purchased from Du Pont Canada (Markham, Ont., Canada). Dilazep {*NN'*-bis-[3-(3,4,5-trimethoxybenzoyloxy)-propyl]homopiperazine} was provided by Asta Werke (Frankfurt, Germany), and soluflazine {[3-(aminocarbonyl)-4-(2,6-dichlorophenyl)-4-(4-fluorophenyl)-4-(3-pyridinyl)butyl]-*N*-(2,6-dichlorophenyl)-1-piperazineacetamide,2HCl} and R75231 {2-(aminocarbonyl)-*N*-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis-(4-fluorophenyl)pentyl]-1-piperazineacetamine} were gifts from Dr. H. Van Belle, Janssen Research Foundation (Beerse, Belgium). Other nucleosides and nucleotides, NBTGR and dipyridamole

Abbreviations used: NBTGR, nitrobenzylthioguanosine; R75231, 2-(aminocarbonyl)-*N*-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis-(4-fluorophenyl)pentyl]-1-piperazineacetamide.

{2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-*d*]pyrimidine} were supplied by Sigma. All other compounds were of reagent grade.

Cultivation and isolation of Ehrlich ascites tumour cells

Cells were grown as an intraperitoneal culture in mice (Swiss, male, ~ 30 g) and transferred weekly to new hosts by intraperitoneal inoculation with 0.2 ml of undiluted ascites fluid. At 5–7 days after inoculation, cells were harvested and washed three times in iso-osmotic saline to remove contaminating erythrocytes. The cell pellet was then resuspended in Dulbecco's phosphate-buffered saline (pH 7.35), or an iso-osmotic 'low sodium' buffer (2 mM-Na^+, 230 mM-*N*-methylglucammonium ion) where appropriate. In some cases, cells were depleted of ATP by sequential incubation with rotenone (20 ng/ml; 15 min at 37 °C) and 2-deoxyglucose (2 mM; 15 min at 37 °C). This procedure has been shown to lower the ATP content of these cells by 95% [14].

[^{3}H]Nucleoside uptake

All assays were conducted at room temperature (~ 22 °C). Uptake was initiated by addition of cell suspension (~ 1×10^7 cells/ml) to [^{3}H]substrate layered over a 200 μl cushion of silicone oil/mineral oil (21:4, v/v) in 1.5 ml micro-centrifuge tubes ('zero-*trans*' conditions). Assays were terminated after a defined incubation time (minimum 5 s) by centrifugation of cells through the oil for 10 s at 12000 ***g***. The supernatant and oil were removed, and the cell pellets were digested with 1 M-NaOH for ~ 16 h at room temperature, and then analysed for ^{3}H content by standard liquid-scintillation-counting techniques in 5 ml of Beckman Ready-Safe scintillation cocktail. The estimated time required to pellet the cells through the oil layer (2 s) is included in all reported incubation times.

Uptake data are presented as intracellular [^{3}H]substrate concentrations (pmol/μl of intracellular volume; μM) after correction for the amount of ^{3}H label present in the extracellular space of the cell pellet. Intracellular and extracellular water volumes of the cell pellets were determined by incubating cells with a combination of [^{14}C]dextran-carboxyl (cell-impermeant) and ^3H_2O for 3 min and then processing the samples as described above. All kinetic values and inhibition constants were derived from the 'mediated' (total minus non-mediated) accumulation of [^{3}H]nucleoside, unless otherwise indicated. Non-mediated uptake was defined as the cellular accumulation of [^{3}H]substrate in the presence of 10 μM-dipyridamole + 10 μM-NBTGR. Initial rates (V_i) of [^{3}H]substrate flux were estimated from the initial slopes of hyperbolic curves fitted (computer-generated) to time-course data. For inhibition studies, cells were usually incubated with inhibitor for 30 min before exposure to [^{3}H]substrate for 22 s; the exceptions were studies involving inhibition of [^{3}H]substrate influx by nucleosides (readily metabolized by cells), where cells were exposed to [^{3}H]substrate and competing nucleoside concurrently. Inhibitor K_i values (inhibition constants) were calculated from the relationship $K_i = IC_{50}/(1 + [S]/K_m)$, where [S] is the assay concentration of substrate, K_m is the Michaelis–Menten constant for substrate transport, and IC_{50} is the concentration of inhibitor required to block influx by 50%, assuming competitive inhibition kinetics.

[^{3}H]Guanosine metabolism

Cells were incubated for 22 s or 182 s with 10 μM-[^{3}H]guanosine and then centrifuged through oil, as described above, in a refrigerated (4 °C) micro-centrifuge. The buffer and oil layers were removed and the cell pellet was immediately solubilized and deproteinized with 100 μl of ice-cold 7% (v/v) $HClO_4$. The precipitate was removed by centrifugation (14000 ***g***; 60 s; 4 °C) and the acid extract was neutralized with 1 M-$NaHCO_3$. This extract (5 μl) was spotted on to a cellulose-coated plate impregnated with a fluorescent indicator (Cellulose F; EM Science, Cherry Hill, NJ, U.S.A.). A mixture of guanosine, GMP, GDP and GTP was co-chromatographed with the samples (R_F values 0.38, 0.11, 0.07 and 0.03 respectively). The solvent system was butan-1-ol/acetone/acetic acid/aq. 5% NH_3/water (7:5:3:3:2, by vol.). After drying, the standards were localized under u.v. light and the sample lanes divided into 0.5 cm horizontal strips. The [^{3}H]guanosine metabolites were extracted from the cellulose plates with 1 ml of water (60 min incubation) and assayed for ^{3}H in 10 ml of Ready Protein (Beckman) scintillation cocktail.

RESULTS AND DISCUSSION

Guanosine inhibition of [^{3}H]uridine influx

Guanosine inhibition of [^{3}H]uridine (100 μM) uptake by ATP-depleted cells was assessed in the absence (total influx) and presence (NBTGR-resistant influx) of 100 nM-NBTGR (Fig. 1); this concentration of NBTGR has been shown to inhibit selectively all NBTGR-sensitive nucleoside uptake by Ehrlich cells [14,16]. Guanosine inhibited the total influx with a K_i of 208 μM, but was 10-fold less effective (K_i = 2.1 mM) as an inhibitor of the NBTGR-resistant component of [^{3}H]uridine influx. The inhibitory potencies of other nucleosides (e.g. adenosine, thymidine) were not affected by incubation of the cells with 100 nM-NBTGR (results not shown). These results confirm that guanosine has a relatively low affinity for the NBTGR-resistant subtype of nucleoside transporter in Ehrlich cells, and suggest that translocation of guanosine through the cell membrane may be mediated primarily by the NBTGR-sensitive transporters.

Effect of cellular ATP depletion on [^{3}H]guanosine and [^{3}H]uridine uptake

Initial studies with [^{3}H]guanosine were conducted as paired experiments with [^{3}H]uridine, by using both ATP-depleted and

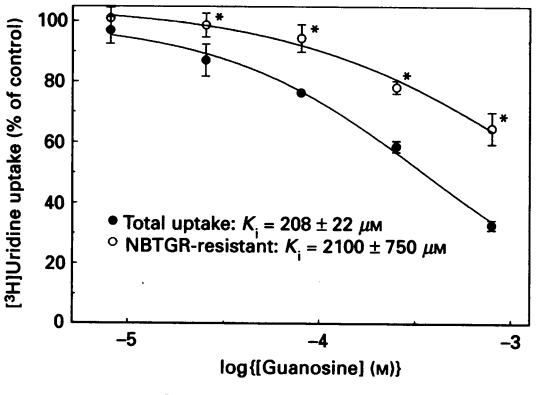

Fig. 1. Inhibition of [^{3}H]uridine influx by guanosine

Guanosine was tested, over a range of concentrations, for its capacity to inhibit the total mediated influx (●) and the NBTGR-resistant influx (○) (cells incubated with 100 nM-NBTGR) of 100 μM-[^{3}H]uridine. Cells were incubated with [^{3}H]uridine and the indicated concentration of guanosine concurrently at 22 °C, as described in the text. Results are presented as a percentage of the accumulation observed in the absence of test inhibitor (control). Each point represents the mean ± S.E.M. from at least five experiments; *indicates a significant difference (Student's *t* test, $P < 0.05$) between the inhibition of NBTGR-resistant influx and the inhibition of total mediated influx. K_i values were derived from these studies by using K_m values for [^{3}H]uridine of 143 μM and 284 μM for the total influx and NBTGR-resistant influx respectively (see [16]).

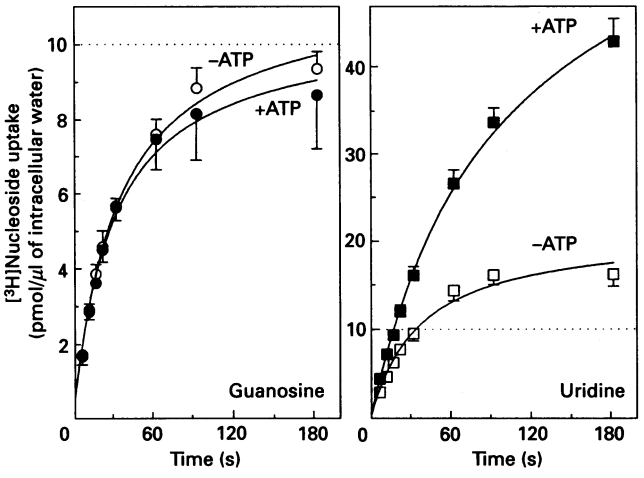

Fig. 2. Comparative time courses for the total accumulation of [3H]guanosine and [3H]uridine metabolites by untreated and ATP-depleted Ehrlich cells

Cells were incubated at 37 °C for 30 min in the absence (●, ■; untreated) or presence (○, □; ATP-depleted) of rotenone/2-deoxyglucose, and then allowed to cool to room temperature (~ 22 °C). These cell preparations were then incubated with either 10 μM-[3H]guanosine (○, ●) or 10 μM-[3H]uridine (□, ■) for the times indicated (abscissa) and the assays terminated as described in the text. The intracellular 3H content is expressed as μM nucleoside equivalents (ordinate) and represents the total accumulation of 3H via both mediated and non-mediated processes. Each point represents the mean ± S.E.M. from at least five experiments. The dotted line represents the expected steady-state intracellular concentration of [3H]nucleoside, based solely on the activity of a non-concentrative facilitated-diffusion system.

Table 1. Comparison of the kinetic constants for the cellular accumulation of [3H]guanosine and [3H]uridine by untreated and ATP-depleted Ehrlich cells

Untreated and ATP-depleted cells were incubated, in parallel, with either 10 μM-[3H]uridine or 10 μM-[3H]guanosine for times ranging from 7 s to 182 s, followed by centrifugation through an oil layer to terminate the assay as described in the text. The data obtained were fitted to a hyperbolic relationship, similar to that shown in Fig. 2, from which the maximum intracellular concentration of [3H]-nucleoside (MAX_i) was extrapolated as well as the half-time to maximum ($t_{\frac{1}{2}}$) and the estimated initial rate of influx (V_i). Each value represents the mean ± S.E.M. from n experiments.

	10 μM-[3H]Uridine ($n = 7$)		10 μM-[3H]Guanosine ($n = 5$)	
	Untreated	ATP-depleted	Untreated	ATP-depleted
MAX_i (μM)	67±5	25±2	12±2	12±1
$t_{\frac{1}{2}}$ (s)	99±9	51±4	36±8	36±5
V_i (pmol/s per μl)	0.68±0.05	0.49±0.04	0.34±0.02	0.32±0.02

untreated (ATP-replete) cells. [3H]Guanosine (10 μM) accumulation by untreated and ATP-depleted cells was similar for time periods of up to 182 s (Fig. 2); only at longer incubation times (> 5 min) were significantly higher cellular accumulations of 3H derived from [3H]guanosine observed in the ATP-replete cells (results not shown). Therefore cellular metabolic activity did not appear to interfere with the determination of the kinetic parameters of guanosine transport (Table 1), as derived from hyperbolic curve fits to time-course data such as those shown in Fig. 2. These data contrast sharply with those obtained with [3H]uridine (see Table 1). Even at incubation times as short as 22 s, the cellular accumulation of 10 μM-[3H]uridine was significantly higher in the ATP-replete Ehrlich cells (Fig. 2), reflecting intracellular trapping of radiolabel as uridine polyphosphates [16].

An interesting, but unexplained, phenomenon observed when using [3H]uridine is that ATP-depleted Ehrlich cells concentrate the unmetabolized nucleoside by 2–3-fold over extracellular levels (Fig. 2, Table 1). This phenomenon cannot be attributed to intracellular trapping as uridine polyphosphates, nor can it be explained entirely by the activity of an ion-dependent concentrative transporter [16], and was most evident at low substrate concentrations (e.g. < 20 μM). It does, however, appear to be particular to [3H]uridine, since in the present study the intracellular concentration of [3H]guanosine (in ATP-depleted cells) did not exceed the expected steady-state level at any of the concentrations tested.

Analysis of the intracellular metabolites of [3H]guanosine showed that Ehrlich cells (ATP-replete) do metabolize [3H]guanosine to guanosine polyphosphates; however, the rate of [3H]guanosine metabolism was significantly slower than that observed for [3H]uridine. After a 22 s incubation with ATP-replete cells, less than 15% of the [3H]guanosine was phosphorylated, compared with 73% for [3H]uridine. Even after 182 s, when more than 85% of the accumulated [3H]uridine was phosphorylated, less than 50% of the intracellular [3H]guanosine was metabolized to its polyphosphates. The intracellular phosphorylation of both [3H]uridine and [3H]guanosine was prevented completely by ATP depletion of the Ehrlich cells.

These results indicate that membrane translocation was the rate-limiting step in the cellular uptake of [3H]guanosine during the time frame of these experiments, and that reasonable estimates of the transport kinetics of [3H]guanosine may be obtained by using ATP-replete, metabolically active, cells, whereas kinetic studies using [3H]uridine must be conducted in ATP-depleted (poisoned) cells. In this regard, [3H]guanosine is similar to [3H]formycin B, which has recently gained popularity as a non-metabolized substrate for the nucleoside transporter [4,6,17,18]. However, unlike formycin B, guanosine is a physiologically relevant, endogenous, nucleoside.

Inhibition of [3H]guanosine uptake

Previous studies have established that both NBTGR-resistant ($K_i > 1$ μM) and NBTGR-sensitive ($K_i < 1$ nM) transporters mediate the uptake of [3H]uridine by Ehrlich cells [14,16]. To assess the relative contributions of these transport systems to the cellular accumulation of [3H]guanosine, the uptake of both [3H]uridine (100 μM) and [3H]guanosine (100 μM) was determined, in parallel, in ATP-depleted cells, in the presence of a range of concentrations (10 pM–1 μM) of NBTGR. NBTGR only partially inhibited the transporter-mediated accumulation of each [3H]substrate in a similar concentration-dependent manner (Fig. 3). However, the amount of NBTGR-resistant [3H]guanosine influx (13% of total uptake) was significantly less than the amount of NBTGR-resistant [3H]uridine influx (23% of total uptake). The IC_{50} values for NBTGR inhibition of the 'NBTGR-sensitive' component of [3H]uridine and [3H]guanosine influx were almost identical (Table 2).

Dilazep has also been reported to distinguish between the NBTGR-sensitive and NBTGR-resistant nucleoside transporters [3,10,16,19]. In the present study, dilazep inhibited the uptake of 100 μM-[3H]guanosine in a biphasic manner (Fig. 3), with IC_{50} values of 2.3 nM and 1.2 μM for the high- and low-affinity components respectively (Table 2). Furthermore, as was observed with NBTGR, the proportion of [3H]guanosine influx resistant to inhibition by dilazep was significantly less than the dilazep-

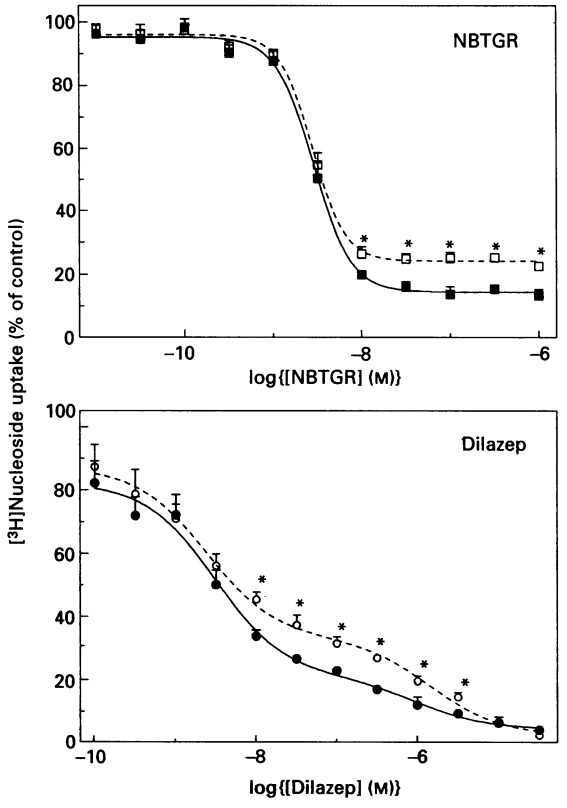

Fig. 3. Inhibition of [³H]guanosine and [³H]uridine influx by NBTGR and dilazep

ATP-depleted Ehrlich cells were incubated with the indicated concentrations of NBTGR (top panel) or dilazep (bottom panel) for at least 15 min and then exposed to 100 μM-[³H]guanosine (●, ■) or 100 μM-[³H]uridine (○, □) for 22 s. The results are presented as a percentage of the [³H]nucleoside accumulation observed in the absence of inhibitor (control). Each point represents the mean ± S.E.M. from at least five experiments; *indicates that the cellular accumulation of [³H]uridine was significantly greater (Student's t test, $P < 0.05$) than that of [³H]guanosine in the presence of these concentrations of inhibitor.

Table 2. Inhibition of [³H]guanosine and [³H]uridine influx by NBTGR and dilazep

Ehrlich cells were preincubated with a range of concentrations of each inhibitor at 22 °C for at least 15 min and then incubated with [³H]uridine (100 μM) or [³H]guanosine (10 μM or 100 μM) for 22 s. Cells were separated from the incubation medium by centrifugation through an oil layer, and assayed for ³H content as described in the text. The data obtained were best described by a two-component concentration–effect relationship (see Fig. 3); IC_{50} values were derived for both the high-affinity (IC_{50}-high) and low-affinity (IC_{50}-low) components. The '% resistant' value represents the proportion of the [³H]nucleoside influx that was relatively insensitive to inhibition. Each value represents the mean ± S.E.M. from at least five experiments.

Inhibitor		[³H]Guanosine 10 μM	[³H]Guanosine 100 μM	[³H]Uridine 100 μM
NBTGR	IC_{50}-high (nM)	1.8 ± 0.3	2.5 ± 0.4	2.4 ± 0.4
	IC_{50}-low (μM)	> 1	> 1	> 1
	% resistant	7 ± 1	13 ± 2	23 ± 2
Dilazep	IC_{50}-high (nM)	2.7 ± 0.4	2.3 ± 0.5	1.4 ± 0.6
	IC_{50}-low (μM)	1.2 ± 0.2	1.2 ± 0.4	1.8 ± 0.6
	% resistant	13 ± 1	21 ± 2	36 ± 3

Table 3. Inhibition of [³H]guanosine and [³H]uridine influx by a variety of nucleoside-transporter substrates and inhibitors

Cells were incubated with a range of concentrations of test inhibitor and [³H]guanosine or [³H]uridine for 22 s, then the reaction was terminated as described in the text. IC_{50} values were derived from sigmoid curves plotted to data as shown in Fig. 4. Inhibition constants (K_i) were calculated from the IC_{50} values by using the relationship $K_i = IC_{50}/(1 + [S]/K_m)$, where [S] = 10 μM or 100 μM, and K_m = 142 μM or 143 μM for guanosine or uridine influx respectively. Each value represents the mean ± S.E.M. from at least five experiments.

Inhibitor	K_i (μM) [³H]Guanosine	K_i (μM) [³H]Uridine
2-Chloroadenosine	22 ± 2	32 ± 3
Adenosine	23 ± 2	52 ± 2
2′-Deoxyadenosine	45 ± 6	42 ± 4
Inosine	52 ± 12	95 ± 7
Uridine	75 ± 6	(143 ± 31)*
Guanosine	(142 ± 11)*	208 ± 22
2′-Deoxyguanosine	207 ± 16	346 ± 42
2′-Deoxycytidine	229 ± 41	273 ± 25
Thymidine	233 ± 38	173 ± 15
Cytidine	353 ± 41	569 ± 63
Dipyridamole	118 ± 17 nM	111 ± 28 nM
R75231	237 ± 56 nM	289 ± 37 nM
Soluflazine	781 ± 31 nM	828 ± 40 nM

* K_m value for transporter-mediated influx of [³H]nucleoside.

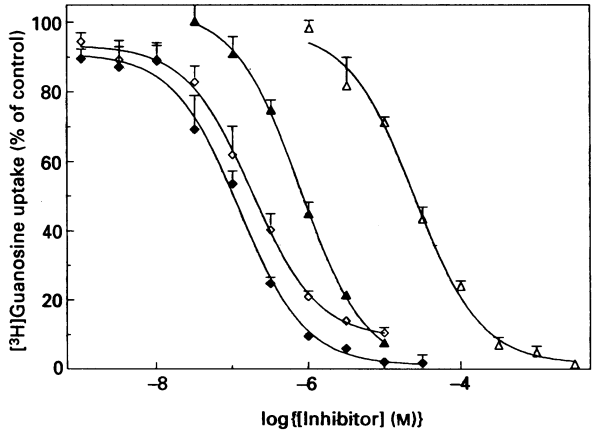

Fig. 4. Inhibition of transporter-mediated [³H]guanosine influx by adenosine (△), soluflazine (▲), R75231 (◇) and dipyridamole (◆)

Ehrlich cells were incubated with 10 μM-[³H]guanosine for 22 s in the absence or presence of the indicated concentrations (abscissa) of inhibitors. Transporter-mediated accumulation of [³H]guanosine in the presence of inhibitor is shown as a percentage of the mediated accumulation observed in the absence of inhibitor (ordinate). Each point represents the mean ± S.E.M. from five experiments.

resistant proportion of [³H]uridine influx, and the relative amount of dilazep-resistant influx was dependent on the [³H]guanosine concentration used (Table 2). It is also of interest that, under identical conditions, with either [³H]uridine or [³H]guanosine the relative amount of dilazep-resistant influx was consistently larger than the amount of NBTGR-resistant influx (Table 2).

A variety of other established nucleoside-transport inhibitors and substrates were tested for their capacities to inhibit the accumulation of [³H]guanosine by ATP-replete Ehrlich cells (Table 3, Fig. 4). In general, the potencies of these agents for inhibiting [³H]guanosine influx were comparable with, or greater

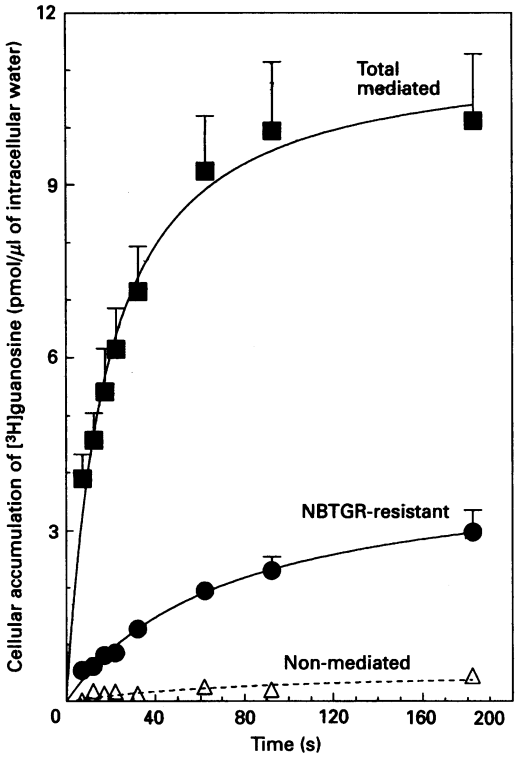

Fig. 5. Time course of 10 μM-[3H]guanosine accumulation by ATP-replete Ehrlich cells

Cells were incubated with 10 μM-[3H]guanosine in the absence and presence of 100 nM-NBTGR or 10 μM-dipyridamole/NBTGR (non-mediated influx) for the times indicated (abscissa), and the assays were then terminated as described in the text. The total transporter-mediated influx (■) and the NBTGR-resistant mediated influx (●) of [3H]guanosine (pmol/μl of intracellular water; ordinate) were calculated as the total cellular accumulation, determined in either the absence or presence of 100 nM-NBTGR respectively, minus the non-mediated component (△). Each point represents the mean ± S.E.M. from five experiments.

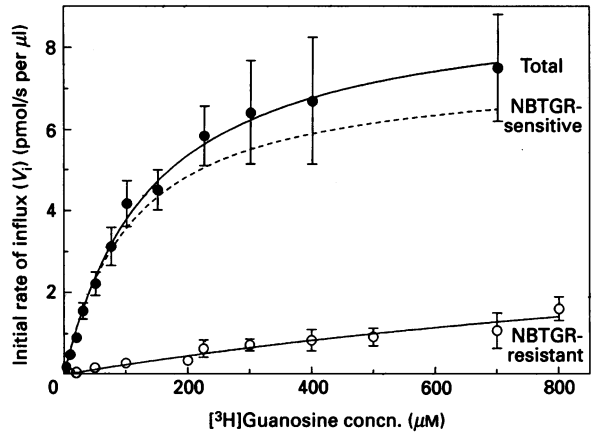

Fig. 6. Concentration-dependence of transporter-mediated [3H]guanosine accumulation by Ehrlich cells

Time courses for the cellular accumulation of a range of concentrations of [3H]guanosine were constructed, as described in the text, by using untreated (ATP-replete) cells in the absence (Total) and presence (NBTGR-resistant) of 100 nM-NBTGR. Initial rates of influx were estimated from the initial portions of hyperbolic curves fitted to the mediated-uptake data (see Fig. 5) and plotted against the [3H]guanosine concentration employed, to obtain the hyperbolic relationships shown. Each point represents the mean ± S.E.M. from at least five experiments. Michaelis–Menten constants (below) were derived from these experimental data by computer-assisted analysis of the non-linear curve fits. K_m and $V_{max.}$ estimates for the NBTGR-sensitive component of [3H]guanosine influx were derived from the hyperbolic relationship (dashed line) obtained by subtraction of the 'Total' and 'NBTGR-resistant' curves.

	K_m (μM)	$V_{max.}$ (pmol/s per μl)
Total	142 ± 11	9.2 ± 0.3
NBTGR-resistant	1780 ± 580	3.7 ± 0.5
NBTGR-sensitive	110	7.5

than, their potencies for inhibiting [3H]uridine accumulation. Dipyridamole was the most potent, followed by lidoflazine derivatives R75231 and soluflazine, and 2-chloroadenosine. All of these agents had pseudo-Hill coefficients for inhibition of [3H]guanosine influx that were not significantly different from unity. Of the endogenous nucleosides tested, adenosine was the most potent inhibitor of [3H]guanosine influx, with a K_i value of 23 μM, followed by 2′-deoxyadenosine, inosine, uridine, 2′-deoxyguanosine, 2′-deoxycytidine, thymidine and cytidine. All of these nucleosides also had pseudo-Hill coefficients, for inhibiting 10 μM-[3H]guanosine influx, that were not significantly different from unity. This contrasts with the results obtained with [3H]uridine as the substrate, where guanosine, 2′-deoxyguanosine, cytidine and 2′-deoxycytidine had pseudo-Hill coefficients significantly less than unity for the inhibition of [3H]uridine influx [16].

Kinetics of [3H]guanosine influx

Time courses for the transporter-mediated (total and NBTGR-resistant) and non-mediated accumulation of a range of concentrations of [3H]guanosine by ATP-replete cells were constructed by using incubation times ranging from 7 to 302 s. Fig. 5 shows data from a set ($n = 5$) of paired experiments (with or without 100 nM-NBTGR, with or without 10 μM-dipyridamole/NBTGR) using 10 μM-[3H]guanosine. The non-mediated uptake component was linear with incubation time and was similar to that obtained with 10 μM-[3H]uridine (results not shown). The total mediated influx of 10 μM-[3H]guanosine was saturable, with an initial rate of influx (V_i) of 0.598 ± 0.075 pmol/s per μl, which is similar to the rate of [3H]uridine (10 μM) influx in ATP-depleted cells (~ 0.5 pmol/s per μl; see Table 1 and [16]). Preincubation of the cells with 100 nM-NBTGR resulted in a 90% decrease in the rate of transporter-mediated influx of 10 μM-[3H]guanosine (NBTGR-resistant $V_i = 0.057 \pm 0.003$ pmol/s per μl), and this rate was significantly slower than the rate of NBTGR-resistant [3H]uridine (10 μM) accumulation by ATP-depleted Ehrlich cells ($V_i = 0.106 \pm 0.011$ pmol/s per μl; see [16]).

Similar initial-rate estimates were obtained for a range of concentrations of [3H]guanosine (1–800 μM) with untreated (ATP-replete) Ehrlich cells. It is important to note that alkaline solutions were required to dissolve guanosine at concentrations above 300 μM; the pH of a 800 μM solution of guanosine was 7.9. Mediated uptake data derived from these studies were used to construct the relationships shown in Fig. 6. The total transporter-mediated uptake process for [3H]guanosine appeared saturable, with a $V_{max.}$ of 9.2 pmol/s per μl and a K_m of 142 μM. This K_m value is similar to the K_i calculated for guanosine inhibition of [3H]uridine influx (208 ± 22 μM; see Fig. 1), and is almost identical with the K_m (143 μM) for the total mediated influx of [3H]uridine [16]. In contrast, the NBTGR-resistant transport component was not saturable over the concentration range tested, but the data could be fitted to a hyperbolic relationship (correlation coefficient

= 0.982) thereby allowing the estimation, by extrapolation, of K_m and $V_{max.}$ values of 1780 μM and 3.7 pmol/s per μl respectively. This K_m value is comparable with the K_i determined for guanosine inhibition of NBTGR-resistant [^{3}H]uridine influx (2100 μM; see Fig. 1), and is almost 10 times the K_m of [^{3}H]uridine for the NBTGR-resistant system (284 μM; see [16]). Estimates of the Michaelis–Menten constants for [^{3}H]guanosine uptake via the NBTGR-sensitive transporter were derived by subtracting the NBTGR-resistant-uptake values from the total-uptake values shown in Fig. 6, yielding K_m and $V_{max.}$ values of 110 μM and 7.5 pmol/s per μl respectively. Therefore [^{3}H]guanosine had a 16-fold lower affinity for the NBTGR-resistant nucleoside transporter relative to the NBTGR-sensitive system.

Na^+-dependent nucleoside influx

Two types of NBTGR-resistant nucleoside transport mechanisms have been described in mammalian cells, a non-concentrative equilibrative system and a concentrative transporter dependent on an inward Na^+ gradient. However, replacing Na^+ with the impermeant *N*-methyl glucammonium ion had no significant effect on the accumulation of [^{3}H]guanosine by Ehrlich cells, even under conditions where over 80 % of the equilibrative transport was blocked with 100 nM-NBTGR (results not shown); inhibition of the equilibrative transport systems has been shown to enhance the cellular accumulation of nucleosides in systems which possess a Na^+-dependent transport mechanism [6,18]. Therefore the cellular accumulation of [^{3}H]guanosine was not dependent on a Na^+ gradient, indicating that Ehrlich cells do not possess the purine-selective Na^+-dependent transporter (N1 subtype; see [5] for nomenclature) that has been characterized elsewhere. These cells may, however, express the thymidine-selective form of Na^+-dependent transporter (N2 subtype; see [5] for nomenclature), as indicated by the Na^+-dependence of a proportion of the [^{3}H]uridine accumulation observed in a previous study [16].

Conclusions

[^{3}H]Guanosine is transported into Ehrlich cells primarily via the NBTGR-sensitive facilitated nucleoside transport system. [^{3}H]Guanosine does not appear to be a substrate for the Na^+-linked concentrative transporter in Ehrlich cells, and it also has a relatively low affinity for the NBTGR-resistant equilibrative transporter. Furthermore, the kinetic parameters for the cellular uptake of [^{3}H]guanosine were not affected by intracellular metabolism within the first 3 min of incubation, making it feasible to conduct transport studies using [^{3}H]guanosine in metabolically active cells. These characteristics, and the fact that guanosine is an endogenous nucleoside, make [^{3}H]guanosine an attractive substrate for use in studies focused on the NBTGR-sensitive facilitated nucleoside transport system of mammalian cells.

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