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S-SUBSTITUTED DERIVATIVES OF 6-MERCAPTOPURINE RIBOSIDES INTERACT BOTH WITH THE TRANSPORT AND METABOLIC PHOSPHORYLATION OF URIDINE BY VIRUS-TRANSFORMED HAMSTER FIBROBLASTS

E. SHOHAMI *, N. KANNER and R. KOREN **

Department of Biological Chemistry, The Hebrew University, Jerusalem (Israel)

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Summary

The uptake of uridine by mammalian cells consists of transport of uridine across the plasma membrane followed by its metabolic conversion, mainly by phosphorylation.

S-substituted aromatic derivatives of 6-mercaptopurine ribosides are potent inhibitors of the nucleoside uptake systems in human erythrocytes and in mammalian cells in culture and have been studied extensively.

We present here a theoretical analysis which enables one to decide whether transport of metabolites, their metabolic trapping within the cell, or both, are susceptible to inhibition. This analysis was applied in the study of the effect of some inhibitors on uridine and cytosine-β-D-arabinoside uptake by transformed Nil-8 cells. It was found that in Nil-SV cells, both transport and metabolic conversion are susceptible to inhibition by nitrobenzylmercaptoinosine and by dansylaminoethylmercaptoguanosine. Nitrobenzylmercaptoinosine displays inhibition constants of 20 and 7 nM for transport and phosphorylation, respectively, while for dansylaminoethylmercaptoguanosine the inhibition constants are 1.8 and 0.6 μM, respectively, for the same processes.

Cytosine-β-D-arabinoside is a synthetic nucleoside which is not metabolizable in Nil cells. Its uptake properties are determined by the transport mechanism alone. The transport of this nucleoside into Nil-SV cells is inhibited by nitro-

* Present address: Department of Neurology, Hadassah Medical Centre, Jerusalem.

** To whom correspondence should be addressed at (present address): Department of Pharmacology, The Hebrew University, Hadassah Medical School, Jerusalem.

Abbreviation: NBMI, *S*-(4′-nitrobenzyl)-6-mercaptoinosine; DAMG, *S*-(*N*-dansylaminoethyl)-6-mercaptoguanosine.

benzylmercaptoinosine and the inhibition constant found is approx. 5 times greater than that for uridine.

Introduction

One can arrest growth of cells in culture by reducing the serum content of their growth medium. These cells are quiescent and may be stimulated to redivide by the addition of fresh serum. It has been shown [1—3] that uridine uptake by quiescent cells is stimulated by the addition of serum or insulin. This stimulation occurs within minutes after exposing the cells to fresh medium containing the activator, and seems to be a cooperative process [3]. As the enhancement of the rate of uridine uptake is one of the first events occurring when quiescent cells are stimulated to grow, the elucidation of the molecular mechanism of the regulation of this process would be of great interest.

The uptake of metabolizable pyrimidine nucleosides by mammalian cells proceeds in two successive steps: (i) transport across the plasma membrane via a facilitated diffusion mechanism, followed by (ii) metabolic conversion. The main metabolic pathway is phosphorylation [4] and the nucleotides thus formed are trapped within the cells.

The kinetic differentiation between transport and metabolic trapping is of great significance, especially since it has been shown that the process susceptible to serum stimulation is the metabolic trapping, rather than transport of uridine. This fact was established both for 3T3 cells [5,6] and for Nil-8 cells [7].

When a non-metabolizable substrate is taken up by cells (e.g., cytosine-β-D-arabinoside by Nil-8 cells), the total uptake is determined by the transport across the plasma membrane. On the other hand, when a metabolizable substrate (e.g., uridine) is taken up, metabolic trapping within the cells affects the shape of the uptake curve. It has recently been shown [7,8] that the kinetic parameters of both steps can be obtained by analyzing data at short and long uptake times. This method is based on the following considerations:

(i) When transport is faster than metabolic trapping, the uptake rate at short times is determined by the properties of the transport system and the uptake rate at long times reflects the rate of metabolic trapping. (ii) The distinction between these two steps becomes more evident when metabolic trapping is reduced: (the use of ATP-depleted cells as well as kinase-deficient mutants for that purpose was reported previously, see Refs. 6 and 9).

A kinetic analysis of the uptake process in the presence of inhibitors may provide an answer to the question as to whether it is transport, metabolic trapping or both steps that are susceptible to inhibition.

Nitrobenzylmercaptoinosine (NBMI), dansylaminoethylmercaptoguanosine (DAMG) and related compounds are potent inhibitors of nucleoside transport in human erythrocytes [10—15] and of nucleoside uptake in cells in culture [16—20]. It has been suggested by Cass and Paterson [13] and by Eilam and Cabantchik [16] that the inhibitor affects the uptake of uridine via an allosteric mechanism. It has been shown that the inhibitor, DAMG, binds to a site dinstinct from that of uridine on the human erythrocyte [15]. Thus, the inhibi-

tion is probably not caused by mere displacement of the substrate from its binding site on the carrier protein.

Paterson et al. [18] studied the phosphorylation of thymidine and uridine in cell-free extracts in the presence of the inhibitor, NBMI. The phosphorylation rate was unaffected, even at inhibitor concentrations in excess of those that effectively block nucleoside uptake in intact cells. These data suggest the possibility that the effect of the inhibitor on the metabolic trapping of uridine within the cell is lost upon cell disruption.

The study by Goldenberg and Stein [21] is illustrative in this context. They report an in situ increase in apparent affinity for ATP of the uridine phosphorylation system in serum-stimulated 3T3 cells, but they found no enhanced affinity in a cell-free extract prepared from the same activated cells.

The present investigation shows that nitrobenzylmercaptoinosine and dansylaminoethylmercaptoguanosine inhibit both transport and metabolic trapping of uridine in Nil-SV cells.

Theory

The uptake of a metabolite consists of two consecutive events, transport across the plasma membrane, followed by metabolic conversion and trapping [7].

We assume here that the transport occurs via a facilitated diffusion system, obeying the kinetics of a simple carrier [22] and that the kinetics of trapping obey the Michaelis-Menten equation:

$$v_{\text{trap}} = \frac{V^{\text{trap}} \cdot S_2}{K_m^{\text{trap}} + S_2} \tag{1}$$

where S_2 is the concentration of the free substrate inside the cell, and V^{trap} and K_m^{trap} are the Michaelis-Menten parameters for the trapping reaction. At the commencement of an uptake experiment, S_2 equals zero and the uptake rate is determined by the capacity of the transport system only and is given by:

$$v_{\text{trans}} = \frac{V^{\text{trans}} \cdot S_1}{K_m^{\text{trans}} + S_1} \tag{2}$$

where S_1 is the external substrate concentration, and V^{trans} and K_m^{trans} are the Michaelis-Menten parameters for the transport system.

At long uptake times, the uptake curve is linear, since a steady state has been reached, and the net transport rate equals the rate of trapping.

Thus, two separate linear regions can be distinguished in a typical curve of metabolite uptake. At short times, transport across the cell membrane is rate-limiting and the uptake curve is linear as long as the internal free substrate concentration is significantly lower than the external one. Over long times, after the free substrate in the cell has reached a steady-state concentration, the uptake curve is again linear. In general, the slopes of the two linear regions are different, but where the metabolic trapping reaction is very fast relative to transport the transport step is rate-limiting for the whole duration of the uptake experiment and the uptake curve is linear over the whole time range.

Heichal et al. [7] have shown in detail how the parameters for both trans-

port and metabolic conversion can be obtained by uptake experiments performed over a wide range of times and substrate concentrations. A special case discussed by them is of relevance to our work, namely, where the external substrate concentration reaches a limiting low value ($S_1 << K_m^{trans}, K_m^{trap}$). Here:

$$\lim_{S_1 \to 0} v_{\text{steady state}} = \frac{S_1}{\frac{K_m^{trap}}{V^{trap}} + \frac{K_m^{trans}}{V^{trans}}} = \frac{S_1}{\frac{1}{k_{trap}} + \frac{1}{k_{trans}}} \tag{3}$$

The rate constant k, is defined as the ratio V/K_m. The rate is linear with substrate concentration. Under the same experimental conditions, the initial uptake rate will be given as:

$$\lim_{S_1 \to 0} v_{\text{initial}} = \frac{S_1}{\frac{K_m^{trans}}{V^{trans}}} = \frac{S_1}{\frac{1}{k_{trans}}} \tag{4}$$

The ratio (R) between the initial slope of the uptake curve (short times) and the slope at steady state (long times) is given by the following equation:

$$R = \frac{v_{\text{initial}}}{v_{\text{steady state}}} = \frac{\frac{1}{k_{trans}} + \frac{1}{k_{trap}}}{\frac{1}{k_{trans}}} = 1 + \frac{k_{trans}}{k_{trap}} \tag{5}$$

Let us now consider an inhibitor of uptake which affects only the transport component. As inhibitor concentration increases, k_{trans} decreases, so that R decreases and approaches unity. An additional test can be applied in order to distinguish between the effect of inhibitors on transport and metabolic trapping.

The rate of some metabolic conversion reactions can be reduced without a change in the transport rate. An appropriate example is the reduced phosphorylation capacity of ATP-depleted cells. This depletion causes a reduction in the rate of conversion of nucleosides to nucleotides.

Eqn. 3 gives the expression for the uptake rate at steady state for a limitingly low substrate concentration. This is easily converted to:

$$\lim_{S_1 \to 0} v_{\text{steady state}} = \frac{S_1 k_{trap}}{1 + \frac{k_{trap}}{k_{trans}}}$$

The only term which is affected by the addition of an inhibitor to the transport step is the second term in the denominator. It is apparent, therefore, that when k_{trap} is reduced by inhibition of the metabolic conversion, the slope of the uptake curve at long times becomes less sensitive to the addtion of an inhibitor specific to the transport component, since the ratio k_{trap}/k_{trans} becomes small relative to unity.

In this section we have presented two criteria which must be fulfilled to

permit us to conclude that an inhibitor affects only the first step of the uptake, namely transport across the plasma membrane.

a. At low external substrate concentrations, the ratio between the slopes of the uptake curve at short times and at steady state should decrease and approach unity as the inhibitor concentration is raised.

b. When the rate of metabolic conversion is reduced, the slope of the uptake curve at long times should become less sensitive to the effect of the inhibitor.

If either of these two criteria is not fulfilled one may conclude that both transport and metabolic trapping within the cells are affected by the inhibitor.

Methods

Cell culture. Nil-8 is a clone of golden hamster fibroblasts. Nil-HSV is the transformed line derived from Nil-8 by infection with murine sarcoma virus. The cells were a gift from the late Dr. I. MacPherson of the Imperial Cancer Research Fund, London, U.K. The cells were grown and maintained in Dulbecco's modified Eagle's Medium (Biolab, Jerusalem) to which 10% of newborn calf serum, antibiotics and glutamine (Biolab, Jerusalem) had been added. The cells were grown at 37°C in a CO_2-enriched, humidified atmosphere.

Uptake experiments. Cells were subcultured in 35-mm plastic petri dishes (Nunc, 4000 Riskilde, Denmark) at a density of $5 \cdot 10^5$ cells/dish and were used 2—3 days later. Before uptake experiments, the cells were incubated in a solution containing the inhibitor at a desired concentration in phosphate-buffered saline, pH 7.4 containing: NaCl, 8 g/l; KCl, 0.2 g/l; $Na_2HPO_4 \cdot 12\ H_2O$, 2.9 g/l; KH_2PO_4, 0.2 g/l; $CaCl_2 \cdot 2\ H_2O$, 0.1 g/l; $MgCl_2 \cdot 6\ H_2O$, 0.1 g/l. Incubation times were 10 min at room temperature for NBMI and 30 min at 37°C for DAMG, unless otherwise specified.

Uptake of uridine and of cytosine-β-D-arabinoside into acid-soluble pools of Nil-SV cells was studied as a function of time at various concentrations of the inhibitors. At time zero, a 0.5 ml solution, of either [5-^{3}H]uridine (Israel Nuclear Research Centre, Negev) at 2 μCi/ml or [^{3}H]cytosine-β-D-arabinoside (Radiochemical Centre, Amersham, U.K.) at 4 μCi/ml in phosphate-buffered saline was added to each dish. The dishes were kept for the desired period of time in a 20°C water bath. The uptake was terminated by five quick washes with 2 ml of ice-cold buffer, and 0.75 ml of 5% trichloroacetic acid was added for 20 min at 4°C to extract acid-soluble material. Aliquots of 0.5 ml were taken for liquid scintillation counting [6—8].

6-S-substituted derivatives of mercaptoinosine and mercaptoguanosine (Sigma Chemical Co., St. Louis, MO, U.S.A. and Fluka, Buchs, Schweiz, respectively) were synthesized according to the method of Montgomery et al. [23] using either nitrobenzyl bromide (Aldrich Chemical Co., Metuchen, N.J, U.S.A.) for NBMI or dansylaziridine (Pierce Ltd., Rockford, IL, U.S.A.) for DAMG [15].

Determination of phosphorylation rate in cell-free extracts. 0.6 ml of 15 mM KCl was added to a 9 cm culture dish and the cells were scraped off the dishes by means of a rubber spatula, and then fragmented by seven successive freeze-thaw cycles. The extract was then placed in a bath sonicator for 15 min at 0°C.

To initiate phosphorylation, 25 μl of the cell extract were added to 50 μl

assay medium, the final composition of which was: 5 μM [^{3}H]uridine (15 μCi/ml), 50 mM Tris-HCl (pH 7.4), 150 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, and inhibitor at a desired concentration. The mixture was incubated for 20 min at 37°C, and the reaction was terminated by boiling for 30 s. Aliquots of 40-μl extracts were spotted onto 1.2 cm discs of Whatman DE-81 ion-exchange paper, dried and washed, first with 40 ml of 1 mM ammonium formate, then with 40 ml of water and finally with 40 ml of ethanol. The discs were then dried and the remaining radioactivity was counted in a liquid scintillation counter.

ATP depletion and determination. The culture medium was replaced by approx. 1 ml of phosphate-buffered saline or 10 mM 2-deoxyglucose in the same buffer and incubated at 37°C for 20 min. ATP was extracted in 1 ml of boiling water for 15 min (the temperature was maintained above 75°C) [21]. The ATP concentration was determined by the Luciferase assay [23]. The intracellular ATP concentration was calculated on the basis of the number of cells per dish and the corresponding cell water volume being equal to 2.4 pl/cell [7]. The results were averages of five separate determinations.

Results

Effect of inhibitors on transport of uridine

We have studied the transport of uridine (5 μM) into Nil-SV cells in the presence of various concentrations of either NBMI or DAMG.

Uptake of uridine carried out over short times was markedly reduced in the presence of nanomolar concentrations of NBMI (Fig. 1A). The profile of inhibition, as depicted in the modified Dixon plot [25] (Fig. 1B), was apparently linear up to 50 nM NBMI, although a clear deviation appeared at higher concentrations of inhibitor. The deviation was particularly pronounced when cells were exposed to inhibitor for extended periods of time prior to uptake measurements. In order to obtain the concentration needed to produce 50% inhibition (IC_{50}), we carried out linear-regression analysis on points in the linear range of data. The X-intercept of the line yielded the IC_{50} value which, for NBMI, was approx. 20 nM.

Essentially similar results (Fig. 2A) were obtained with DAMG, despite the fact that the latter was considerable less potent than NBMI. The IC_{50} value for DAMG, as estimated from the Dixon plot in Fig. 2, was approx. 2 μM.

Effect of inhibitors on metabolic conversion of uridine

Uptake of uridine was linear with time between 5 and 15 min. By the beginning of this period, internal uridine concentration had reached steady-state levels either in the presence or absence of inhibitors (see Theory and Ref. 7).

In order to ascertain whether or not the reduction in uridine uptake rate at 'long times' resulted from inhibition of transport alone, we analyzed the data, as explained in Theory. For each concentration of inhibitor, we compared the transport rate (i.e., initial rate) to the uptake rate under steady-state conditions (Table I).

We observed that under all conditions, transport was faster than uptake at steady state, depsite the fact that transport rates were reduced up to 80% by

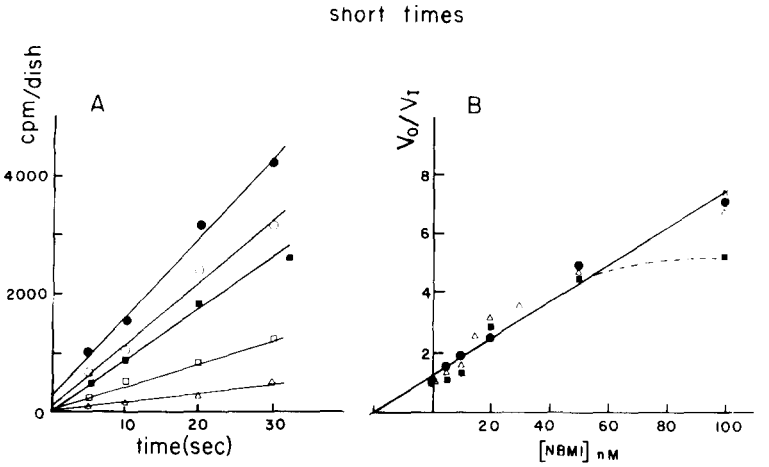


Fig. 1. (A) Typical experiments of uridine uptake at short times by Nil-SV cells in the presence of NBMI. Uridine concentration 5 μM. NBMI concentrations: ●, 0.0 nM; ○, 5 nM; ■, 10 nM; □, 30 nM; △, 100 nM. Cell density, $1.5 \cdot 10^6$ cells/3.5 cm^2 dish. Lines drawn by linear regression. Cells were incubated with the inhibitor for 10 min at room temperature, prior to the uptake measurements. (B) Modified Dixon plot of the data of A and other experiments under various experimental conditions. ●, no pretreatment of cells with inhibitor prior to an uptake experiment. Data combined from two different experiments, one at a cell density of $5 \cdot 10^5$ and the other of $1.3 \cdot 10^6$ cells/dish. △, cells preincubated with the inhibitor for 10 min at room temperature. ■, cells incubated with the inhibitor for 30 min at 37°C. The straight line drawn through the points was obtained by linear regression of a total of 18 points (not all shown).

the presence of inhibitor. The ratio of the rate of transport to the rate of uptake at steady state did not decrease with increasing inhibitor concentration. As explained in Theory, this result indicates that both transport and metabolic trapping are inhibited. Furthermore, the latter is rate-limiting at long uptake

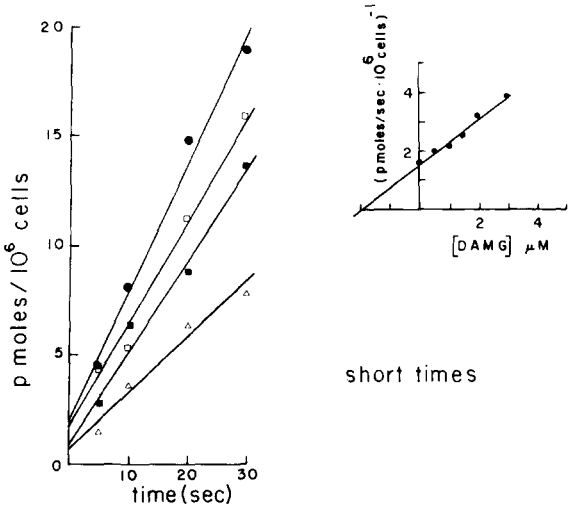


Fig. 2. Uridine uptake by Nil-SV cells in the presence of DAMG, measured at short times. Cells at a density of $1.5 \cdot 10^6$ cells/dish were incubated with the inhibitor for 30 min at 37°C prior to the experiment. Uridine concentration 5 μM and the inhibitor concentrations (in μM): ●, 0.0; □, 1.0 μM; ■, 1.5 μM and △, 3.0 μM. Inset: a plot of $1/v$ vs. inhibitor concentration, from the data shown in the figure, and similar experiments performed with parallel cultures.

TABLE I

EFFECT OF INHIBITORS ON THE RATES OF UPTAKE OF 5 μM URIDINE AT 'SHORT' AND 'LONG' TIMES

DAMG, Dansylaminoethylmercaptoguanosine; NBMI, Nitrobenzylmercaptoinosine. Transport and steady-state rates are expressed in cpm/s.

Inhibitor	Concentration	Transport rate (short)	Steady-state rate ('long')	R = rate 'short'/rate 'long'
DAMG	0 μM	187 ± 11	64 ± 8	2.9 ± 0.5
	1	148 ± 10	52 ± 7	2.9 ± 0.6
	2	96 ± 8	30 ± 5	3.1 ± 0.8
	5	33 ± 2	15 ± 0.5	2.2 ± 0.2
NBMI	1 nM	100 ± 9	53 ± 8	1.9 ± 0.6
	2	89 ± 7	45 ± 2	2.0 ± 0.2
	10	75 ± 9	33 ± 4	2.3 ± 0.5
	30	39 ± 7	12 ± 3	3.2 ± 1.5

times, both in the presence, and in the absence, of inhibitors.

ATP depletion of cells provides an easy means of inhibiting the phosphorylation of a nucleoside. Moreover, it can serve as a test to find out whether or not a certain nucleoside is phosphorylated. The uptake time course of a non-metabolite should not be affected by pretreatment of the cells with 2-deoxyglucose while nucleosides that undergo phosphorylation should be affected. Fig. 3 illustrates the effect on the uptake of uridine and of cytosine-β-D-

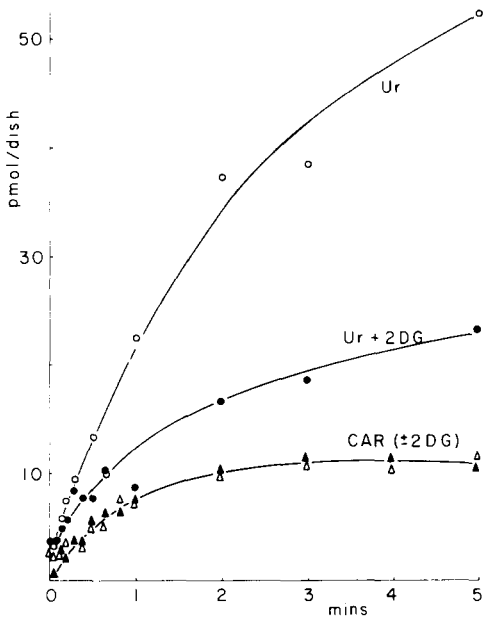


Fig. 3. The effect of 2-deoxyglucose (2DG) on the uptake of uridine (Ur) and cytosine-β-D-arabinoside (CAR) by Nil-8 cells. Cell density was $0.52 \cdot 10^6$ cells/dish. Nucleoside concentration was 10 μM. Cells were incubated for 30 min in phosphate-buffered saline (○,△) or in 50 mM 2-deoxyglucose in the same buffer (●,▲), at 37° C, prior to the experiment.

arabinoside, of preincubation of Nil-SV cells with 50 mM 2-deoxyglucose. It is clear that uridine uptake decrease significantly as a result of such treatment while cytosine-β-D-arabinoside (which does not undergo phosphorylation in these cells [7]) is not affected (under the same conditions). ATP depletion does not affect either the nucleoside transport rate or the equilibrium level reached by a non-metabolite.

The inhibition of uridine uptake by NBMI and by DAMG cannot be attributed to transport alone, since the first criterion (presented in Theory) is not fulfilled. Further support for this conclusiion is given by a set of experiments we shall now describe. Reduction of phosphorylation is achieved by incubating the cells in 2-deoxyglucose. As explained in Theory, if NBMI were only a transport inhibitor, its effect on the uptake rate at long times (at steady state) should be greatly reduced after 2-deoxyglucose treatment.

Table II summarizes the results of uridine uptake experiments at steady state after a combined treatment with 2-deoxyglucose and NBMI. It is apparent that, although uridine trapping is reduced more than 2-fold as a result of 10 mM 2-deoxyglucose pretreatment, the extent of inhibition caused by 10 nM NBMI is only slightly affected. The last two columns of Table II were calculated by using the data of Tables I and II, the Dixon plot of Fig. 1B and Eqn. 3. We assumed (from Fig. 3) that the rate of transport is unaffected by 2-deoxyglucose treatment.

In a control experiment it was established, by means of the Luciferase assay, that pretreatment of Nil-SV cells with 10 mM 2-deoxyglucose reduces the internal ATP concentration from 2.8 ± 0.7 mM to 1.2 ± 0.4 mM (the inernal cell water volume was assumed to be 2.4 pl/cell [7]).

As explained in Theory, the data presented in Table II further strengthen our previous conclusion that metabolic trapping is susceptible to NBMI inhibition.

We have established the fact that NBMI inhibits both transport and phosphorylation of uridine in Nil-SV cells and we proceeded to investigate the detailed mechanism of inhibition.

Kinetic analysis of the uptake data at long times was carried out in the inhibitor concentration range 0—20 nM. The uptake of uridine at various concentrations (10, 20, 50 and 100 μM) was studied in the presence of 0, 5, 10 or 20 nM of NBMI, in the time range 5—15 min. The data were treated in terms of

TABLE II

THE EFFECT OF PRETREATMENT WITH 2-DEOXYGLUCOSE ON THE INHIBITION OF URIDINE (5 μM) TRANSPORT AND TRAPPING BY NBMI

2-DG, 2-deoxyglucose; NBMI, nitrobenzylmercaptoinosine.

Conditions		Rate (pmol/min)		
(2-DG) (mM)	(NBMI) (nM)	$V_{steady\text{-}state}$	V_{trap}	V_{trans}
0	0	30.5	46.5	88.5
10	0	14.2	16.9	88.5
0	10	8.1	9.4	59
10	10	4.8	5.2	59

the Michaelis-Menten equation and plotted as S/V vs. S for each NBMI concentration. The maximal velocity (V) is given by the reciprocal value of the slope and the apparent Michaelis constant (K_m') is given by the ratio of the intercept and the slope of each straight line. The parameters derived by linear regression of the data points are summarized in Table II. There may be a slight decrease in the maximal velocity, but the inhibition is mainly due to an increase in the apparent K_m. The plot of K_m' vs. I yielded a straight line, from which the inhibition constant (K_I) was calculated and found to be 7.2 ± 0.4 nM.

Assuming that the inhibition mode is essentially competitive, we analyzed uptake experiments in the presence of DAMG at long times, in terms of the Dixon equation. The results are shown in Fig. 4. K_i for DAMG was calculated from the X-intercept of the straight line of Fig. 4 with the value K_m = 39 μM (Table III), and was found to be 0.7 ± 0.2 μM.

The effect of NBMI on cytosine-β-D-arabinoside uptake

Cytosine-β-D-arabinoside is a synthetic nucleoside which does not undergo phosphorylation in Nil-SV cells under our experimental conditions (see Fig. 3 and Ref. 7). Thus, the effect of NBMI on the uptake of this substrate reflects the effect on transport alone. The uptake of 5 and 50 μM of cystosine-β-D-arabinoside into Nil-SV cells was studied in the presence of 0 to 200 nM NBMI and the results are shown in Fig. 5. The IC_{50} value for 5 μM cytosine-β-D-arabinoside is approx. 110 nM, which is approx. 5 times greater than that for 5 μM uridine. The IC_{50} value for 50 μM is approx. 150 nM. The fact that one obtains slightly different K_I values at different substrate concentrations can be attributed to a competitive mode of inhibition where the K_m for cytosine-β-D-arabinoside is 250 μM [7].

The effect of NBMI on uridine phosphorylation in cell-free extracts

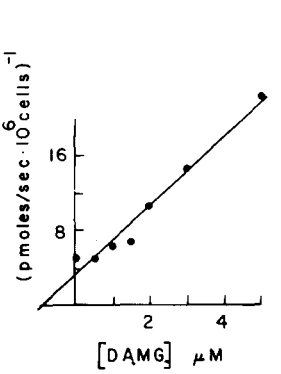
In order to approach an understanding of the mechanism of NBMI action on the uridine phosphorylation system, we proceeded to study this reaction in cell-free extracts. Uridine kinase is responsible for the first and rate-limiting step of uridine phosphorylation within the cells [5]. We felt that by extracting this enzyme from the cells and adding the substrates (uridine, ATP and Mg^{2+}),

TABLE III

KINETIC PARAMETERS FOR THE INHIBITION OF URIDINE UPTAKE AT STEADY STATE BY NBMI

Data for the slope were analyzed in terms of the Michaelis-Menten equation, expressed as S/v vs. S. The apparent K_m values are those in the presence of the inhibitors.

I (nM)	Slope ($\times 10^{-3}$) (pmol/s per dish)$^{-1}$	Intercept {μM/(pmol/s per dish)}	K_m' (μM)	V (pmol/s per dish)
0	4.4 ± 0.07	0.18 ± 0.04	39 ± 7	227 ± 5
5	4.6 ± 0.07	0.31 ± 0.01	67 ± 5	217 ± 1
10	5.0 ± 0.04	0.47 ± 0.04	94 ± 10	200 ± 2
20	5.0 ± 0.07	0.7 ± 0.1	204 ± 5	200 ± 3



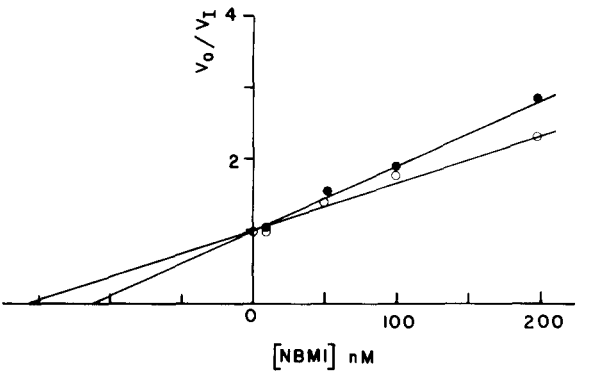


Fig. 4. The effect of DAMG on the rate of uridine uptake at long times (5—15 min) by Nil-SV cells. Experimental conditions were the same as those in Fig. 2.

Fig. 5. The effect of NBMI on the transport rate of cytosine-β-D-arabinoside by Nil-SV cells. Uptake was measured up to 30 s. ●, 5 μM; cytosine-β-D-arabinoside ○, 50 μM cytosine-β-D-arabinoside. The lines of the modified Dixon plot were drawn by linear regression.

as well as the inhibitor NBMI, we might be able to gain some information on the inhibitor's mode of action.

The results of the phosphorylation of uridine in cell-free extracts in the presence of various concentrations of NBMI are presented in Table IV. It seems that the phosphorylation rate is unchanged (or even slightly enhanced) at low concentrations of the inhibitor. A slight inhibitory effect is observed when the concentration of the inhibitor is much higher than the inhibition constant measured for intact cells.

TABLE IV

PHOSPHORYLATION OF URIDINE IN CELL-FREE EXTRACTS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF NITROBENZYLMERCAPTOINOSINE (NBMI)

[NBMI] (nM)	cpm/assay
0	9417
	9940
1	12515
	10964
2	9165
	10482
5	9891
	10438
10	7363
	8688
50	7473
	4766
100	5464
	6743

Discussion

In the course of this study we developed a method which enabled us to study the inhibition effect of some derivatives of 6-mercaptopurine ribosides on nucleoside uptake. The central question was whether this class of compounds affects only transport across the plasma membrane, or further metabolic steps as well. This method is applicable whenever a distinction between transport and metabolic trapping is possible.

The equations developed in Theory are based on the following considerations. In those cases where transport across the plasma membrane is faster than the subsequent metabolic step, the uptake curve describing the accumulation of the substrate within the cell as a function of time is not linear; transport is rate-limiting in the initial part of the curve and metabolic trapping in the later one. Under conditions where transport is specifically inhibited, the shape of the uptake curve changes as a function of inhibitor concentration. If the internal trapping becomes much faster than transport, a strictly linear uptake curve is obtained and its slope yields the rate of transport across the membrane.

When transport occurs via a facilitated diffusion system, the internal concentration of free substrate can never exceed the external one. The internal concentration of free substrate is determined by the relative rates of transport and trapping. The rate of the trapping reaction itself depends on the internal concentration.

In some uptake systems it is possible to reduce metabolic trapping without affecting the rate of transport, resulting in an increase in the free substrate concentration within the cell.

Let us consider the effect on the uptake rate at steady state of an inhibitor specific to transport. This effect is due solely to the reduction of free substrate concentration, and is less pronounced when the internal free substrate concentration is near its maximal value (equal to the external concentration). Thus, we would expect the effect of 'transport inhibition' on the steady-state rate to be less apparent when metabolic trapping capacity is reduced.

We have shown that metabolic trapping is rate-limiting under steady-state conditions for the whole range of inhibitor concentrations studied. This precaution is essential because in those cases where transport is affected to a much greater extent than the trapping reaction, the nature of the rate-limiting step may change with increasing inhibitor concentration.

In this study we have investigated the effect of NBMI and DAMG over a wide range of uptake times and inhibitor concentrations.

The simplest way to explain our data for the inhibition of uridine uptake by NBMI and DAMG is to assume that they act both on transport and on the subsequent metabolic trapping step. This conclusion is based on the fact that the ratio between transport and steady-state uptake does not decrease with increasing inhibition concentration, as well as on the fact that the extent of inhibition is not reduced by depleting the cells of ATP. These data are not consistent with the assumption that the inhibitors are specific to the transport step alone—and one has to conclude that they act upon the second step as well. No indication has been obtained by us [6,7] or by others [9] that the transport step itself has

more than one component. The second step of the uptake has been attributed to the phosphorylation of the nucleoside to the monophosphate. This conclusion is reached by comparison with experiments performed in cell extracts and by the use of kinase-deficient mutants [26], and ATP-depleted cells [9,27]. The positive proof one would like to obtain is the demonstration that the inhibitors indeed affect the rate of conversion of uridine to a higher charged form, in cell-free extracts. The inhibitors do not seem to affect phosphorylation in cell-free extracts (Table IV) under conditions where the reaction rate in intact cells is greatly reduced. The explanation of this phenomenon is not yet known.

In order to be active as anti-cancer reagents, 6-mercaptopurine ribosides have to undergo phosphorylation within the cells, e.g., see Ref. 28. It is possible that the same holds for NBMI before it can act as an inhibitor for the kinase. We tested this hypothesis by incubating the inhibitor with the cells, before preparation of the cell extract. Uridine phosphorylation was assayed and no inhibition was detected as compared with the control. Moreover, it has been shown by Lauzen and Paterson [19] that NBMI does not undergo a metabolic change to any detectable extent after incubation with HeLa cells.

The ratios between the inhibition constants for the transport and trapping steps are remarkably similar for NBMI (20 : 7 nM) and DAMG (2 : 0.7 μM), although the absolute values of these constants differ by two orders of magnitude. This would suggest that the inhibitors do not act separately on both steps.

Wohlhueter et al. [30] found that transport of thymidine into Chinese hamster ovary cells was inhibited by NBMI in a simple non-competitive manner, with an apparent K_i of 1 nM. The inhibition constant reported here is significantly higher. In other studies [14,16,17,29] no clear distinction was made between transport and trapping and the effect of NBMI was studied on the whole process of uptake.

There is some similarity between the inhibition of uridine phosphorylation in intact cells by NBMI and DAMG and the stimulation of uridine phosphorylation in quiescent cells by the addition of serum [5,7]. In both cases the effect observed in the intact cells is lost upon cell disruption [21]. The results presented here may suggest the existence of some coupling mechanism between the uridine trapping system and extracellular signals, which operates in intact cells.

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