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Transport and Metabolism of 9-β-D-Arabinofuranosylguanine in a Human T-Lymphoblastoid Cell Line: Nitrobenzylthioinosine-sensitive and -insensitive Influx

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ABSTRACT

Nitrobenzylthioinosine (NBMPR), dipyridamole, and dilazep, potent inhibitors of nucleoside transport, were found to be ineffective in preventing 9-β-D-arabinofuranosylguanine (ara-G)-induced inhibition of MOLT 4 and CCRF CEM cell growth. ara-G (2.0 μM) was metabolized to 9-β-D-arabinofuranosylguanine 5′-triphosphate in MOLT 4 cells, and the levels of this metabolite were not affected by the presence of 5.0 μM NBMPR in the incubation medium. Permeation of the MOLT 4 cell membrane by ara-G occurred primarily by means of the NBMPR-sensitive nucleoside transport system. However, a residual transport component accounting for 10–20% of the total transport activity was demonstrated in the presence of NBMPR. This component was inhibited by adenine and hypoxanthine but not by dilazep, dipyridamole, or other nucleosides. In contrast, inhibitors of nucleoside transport readily reversed the cytotoxic effect of 7-deazaadenosine (tubercidin) in both MOLT 4 and CCRF CEM cells. The levels of tubercidin 5′-triphosphate formed from 2.0 μM tubercidin in MOLT 4 cells were reduced by 80% in the presence of 5.0 μM NBMPR. The influx of tubercidin into MOLT 4 cells was found to occur primarily by means of the NBMPR-sensitive nucleoside transport system. This same system mediated the transport of ara-G into human erythrocytes.

INTRODUCTION

ara-G,[2] which is metabolized to ara-GTP in several types of cells, has been found to be more inhibitory to the growth of T-lymphoblasts than to B-lymphoblasts (1–3). The cytotoxic activity of ara-G may accrue from the inhibition of DNA polymerase by ara-GTP and/or from the incorporation of ara-G 5′-monophosphate into DNA (1). ara-GTP accumulation has been demonstrated in T-lymphoblastoid cells and in T-cells from patients with acute lymphocytic leukemia but not in B-lymphoblastoid cells (2). This selectivity for T-lymphoblasts has resulted in the consideration of ara-G for chemotherapeutic use in the treatment of T-cell-lymphoproliferative disorders (1). From this perspective, it was of interest to determine whether the inhibitory action of ara-G on T-lymphoblasts could be modulated by inhibitors of nucleoside transport, such as NBMPR. A study of the mode of entry of ara-G in T-lymphoblasts was subsequently initiated in order to understand the resulting observations.

MATERIALS AND METHODS

Materials. [*G*-^{3}H]Tubercidin (17 Ci/mmol) and [8-^{3}H]ara-G (2.5 Ci/mmol) were obtained from Moravek Biochemicals, Inc. [*U*-^{14}C]Sucrose was obtained from NEN Research Products. ara-GTP was purchased from Calbiochem-Behring Corp. RPMI 1640 was obtained from Flow Laboratories. Gentamicin reagent solution and Hepes were purchased from Gibco. Fetal bovine serum was from HyClone Laboratories. Dilazep was provided by Hoffmann-LaRoche Inc. Dipyridamole, NBMPR, adenine, hypoxanthine, tubercidin, guanosine, uridine, and inosine were purchased from Sigma. ara-G was synthesized at the Wellcome Research Laboratories.

Purification of Radioisotopes. [^{3}H]ara-G and [^{3}H]tubercidin were purified to >99% by reversed-phase HPLC using a Waters C_{18}-μBondapak column. Prior to injection, an aliquot of the radioisotope was dried under nitrogen and reconstituted with distilled water. The sample was eluted with a gradient (60 min) of 0–60% acetonitrile in 5 mM ammonium phosphate (pH 5.4) at a flow rate of 1 ml/min. Aliquots of each of 120 fractions (0.5 ml) were monitored for radioactivity by liquid scintillation counting. The peak fractions were pooled, dried under a stream of filtered nitrogen, reconstituted in 50% methanol, and stored at −20°C.

Growth Inhibition Studies. The MOLT 4 and CCRF CEM T-cell lymphoblastic leukemia lines were obtained from the American Type Culture Collection. Cells were maintained in exponential growth in RPMI 1640 supplemented with 10% fetal bovine serum and 1 μg/ml gentamicin at 37°C in a 5% CO_2 humidified atmosphere. For growth inhibition assays, cells (4×10^4 cells/ml in 96-well plates) were incubated for 96 h in the presence of various concentrations of ara-G or tubercidin with 0–5 μM NBMPR. For assays involving reversal of ara-G toxicity by nucleobases, 20 or 100 μM adenine was added to the culture medium. Inhibition of growth was determined by measuring cellular DNA with the propidium iodide assay (4).

Preparation of Human Erythrocytes. Human peripheral blood was obtained from healthy volunteers and erythrocytes were prepared as described previously (5) except that physiological saline containing 10 mM Hepes was used as the medium. The cells were resuspended to yield a final assay hematocrit of 5%.

Kinetics of Nucleoside Influx into Human Erythrocytes. The influx of [^{3}H]ara-G (0.02–0.39 μCi) into human erythrocytes was determined by measuring the cell-associated dpm at appropriate times following the addition of 50 μl of permeant to a 50-μl suspension of erythrocytes. The reaction was terminated by addition of 700 μl of cold 20 mM papaverine and 200 μl of cold *N*-butylphthalate and subsequent centrifugation (13,000 × *g* for 60 s) in a Beckman model B microfuge. Cell pellets were processed as described previously (5). Cell-associated radioactivity was determined in a Packard 2000CA Tri-Carb liquid scintillation counter. Initial velocities of [^{3}H]ara-G influx were determined by linear regression analysis of the slopes of cell-associated ^{3}H *versus* time for data obtained during the linear phase of influx (0–3 s).

Kinetics of Nucleoside Influx into MOLT 4 Cells. MOLT 4 cells were harvested by centrifugation at 350 × *g* for 10 min at room temperature. The cells were resuspended in fresh RPMI 1640 (without serum) containing 10 mM Hepes. An aliquot of the cell suspension was removed for counting and the cells were pelleted by centrifugation. This final pellet was resuspended in a volume of RPMI 1640 (without serum), containing 10 mM Hepes, that yielded a final cell number of 1.0–1.5 × 10^7 cells/assay tube. Influx of [^{3}H]ara-G (0.01–0.89 μCi) or [^{3}H]tubercidin (0.12–3.2 μCi) was determined as described above for human erythrocytes. The initial velocity range for ara-G influx was 0–3 s and 0–2 s for tubercidin influx. Kinetic constants were determined by directly fitting the data to a hyperbola according to the method of Wilkinson (6) and the computer program of Cleland (7).

Identification of [^{3}H]ara-G in Cell Extracts. MOLT 4 cells (1.5×10^7 cells/assay) in 1.5-ml Eppendorf tubes were incubated with 2.0 mM

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[2] The abbreviations used are: ara-G, 9-β-D-arabinofuranosylguanine; ara-GTP, 9-β-D-arabinofuranosylguanine 5′-triphosphate; NBMPR, nitrobenzylthioinosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibitory concentration.

ara-G (1.25 μCi [^{3}H]ara-G) for 5 s at 37°C before addition of 200 μl of warm (37°C) *N*-butylphthalate and centrifugation (13,000 × *g*) for 1 min. The tips of the tubes, containing the cell pellets, were excised and placed in glass centrifuge tubes containing 5 ml of cold 0.5 M perchloric acid. After sonication the tubes were centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatants were neutralized with KOH to pH 6.5–7.5, filtered through glass wool in a Pasteur pipet, and evaporated to dryness in a Buchler Evapo-Mix apparatus. The residue was reconstituted with 300 μl deionized water and stored at −20°C. Cell extracts were analyzed by reversed-phase HPLC (8).

Quantitation of Cellular 5′-Nucleotide Metabolites. MOLT 4 cells (2 × 10^6 cells in 50 ml) were incubated in 75-cm^2 tissue culture flasks for 24 h at 37°C in the presence of 2.0 μM ara-G (12 μCi [^{3}H]ara-G) or 2.0 μM tubercidin (5 μCi [^{3}H]tubercidin), with and without 5.0 μM NBMPR. Cells were harvested by centrifugation (350 × *g*) for 15 min. Pellets were resuspended in 1 ml of fresh medium, placed in Eppendorf tubes, and centrifuged for 1 min (Beckman model B microfuge). The tips of the tubes were severed and placed in glass centrifuge tubes containing 5 ml of cold 0.25 M trichloroacetic acid containing 2.0 μM ITP as a recovery marker. After sonication to disperse the pellet, tubes were centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants were neutralized with 3.5 ml of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane. The aqueous phase was collected and evaporated to dryness on a Buchler Evapo-Mix apparatus and reconstituted with 300 μl of 10 mM Tris-HCl, pH 8.5. Cellular levels of 5′-nucleotides were analyzed by anion-exchange HPLC with isocratic elution (9). The 5′-triphosphate metabolite of [^{3}H]ara-G was quantitated by measuring the amount of radioactivity eluted at the retention time of authentic standards. Each analysis was normalized on the basis of the amount of ITP recovery marker present in each extract.

RESULTS

Effect of Inhibitors of Nucleoside Transport on ara-G Inhibition of MOLT 4 Growth. ara-G inhibited the growth of MOLT 4 cells with an IC_{50} of 5 μM (Fig. 1). Addition of NBMPR, dipyridamole, or dilazep (0.5 or 5.0 μM) to the medium did not alter the cytotoxic effect of ara-G. In contrast, these inhibitors of nucleoside transport markedly shifted the concentration-response curve of cell growth *versus* tubercidin concentration (Fig. 2). The IC_{50} for tubercidin was 0.1 μM in the absence of NBMPR. In the presence of 5.0 μM NBMPR, dipyridamole, or dilazep, the IC_{50} was determined to be 5 μM. Qualitatively similar results were obtained in identical studies using CCRF CEM cells, although the IC_{50} values in the absence of NBMPR were lower (0.1 and 0.03 μM for ara-G and tubercidin, respectively; data not shown). In the presence of NBMPR, adenine (20–100 μM) was found to reverse the ara-G-induced growth inhibition of MOLT 4 and CCRF CEM cells (Table 1).

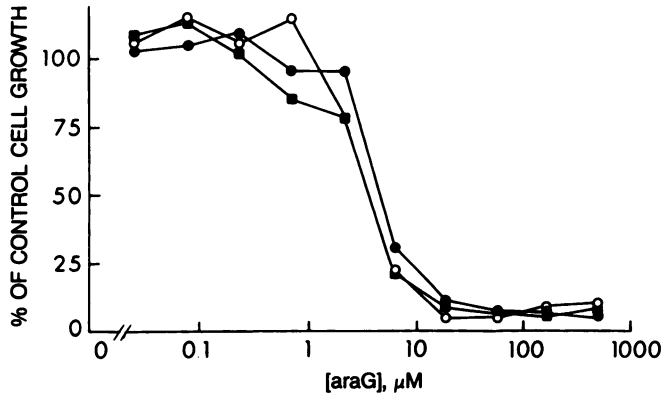

Fig. 1. Effect of NBMPR on ara-G-induced inhibition of MOLT 4 cell growth. MOLT 4 cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 1 μg/ml gentamicin, and varying concentrations of ara-G and NBMPR for 96 h. Cell growth was monitored using the propidium iodide assay for cellular DNA. 0 (●), 0.5 μM (○), and 5.0 μM (■) NBMPR.

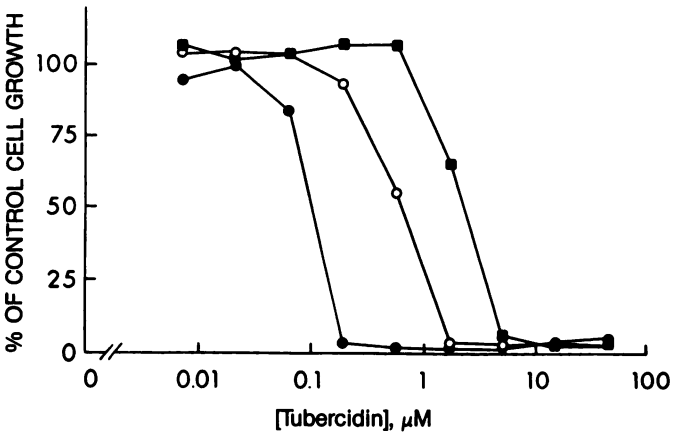

Fig. 2. Effect of NBMPR on tubercidin-induced inhibition of MOLT 4 cell growth. MOLT 4 cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 1 μg/ml gentamicin, and varying concentrations of tubercidin and NBMPR for 96 h. Cell growth was monitored using the propidium iodide assay for cellular DNA. 0 (●), 0.5 μM (○), and 5.0 μM (■) NBMPR.

Table 1 *Effect of adenine on ara-G inhibition of human T-lymphoblastoid cell growth in the presence of 5.0 μM NBMPR: IC_{50} values for ara-G (μM) ± SD*

Cell line	Adenine concentration (μM)		
	0	20	100
MOLT 4	1.1 ± 0.13	1.9 ± 0.04	3.9 ± 0.03
CCRF CEM	0.43 ± 0.02	0.66 ± 0.07	1.1 ± 0.12

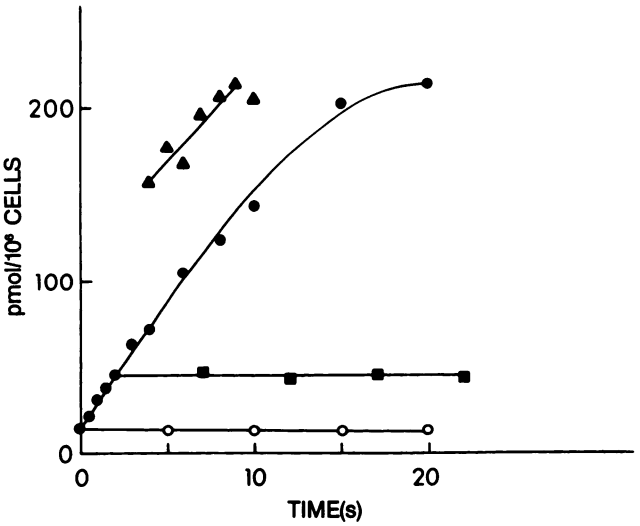

Fig. 3. Verification of the effectiveness of papaverine as a stop agent. The influx of 2 mM [^{3}H]ara-G into MOLT 4 cells (10^7 cells/assay) was determined with the "inhibitor stop" method using 7 volumes of cold 20 mM papaverine to terminate the assay (●); 0- (○) and 2.0-s (■) assay times with up to 20 s holding time before centrifugation are shown. "Oil stop" experiment using centrifugation through *N*-butylphthalate to terminate the assay (▲).

Influx of [^{3}H]ara-G into MOLT 4 Cells. The rate of influx of [^{3}H]ara-G into MOLT 4 cells was investigated with an "inhibitor stop" assay using 7 volumes of cold 20 mM papaverine as the stop agent (10). The use of papaverine was validated in several ways: influx of 2.0 mM [^{3}H]ara-G with time was measured to provide a control rate and was found to be linear for approximately 3 s (Fig. 3). The time between addition of papaverine and the start of centrifugation ("holding time") was 10 s. Two additional series of assays were performed. In the first series, influx was allowed to proceed for 2.0 s. The reaction was then stopped with the cold quench agent and held for 15 to 30 s before centrifugation through oil. In the second series,

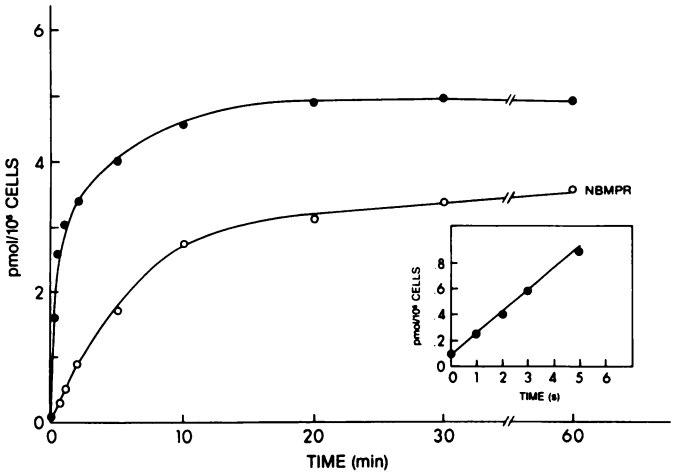

Fig. 4. Time course of [^{3}H]ara-G influx into MOLT 4 cells. The influx of 2.0 μM ara-G into MOLT 4 cells (10^7 cells/assay) was determined as described in "Materials and Methods": 2.0 μM ara-G (●); 2.0 μM ara-G + 2.5 μM NBMPR (○).

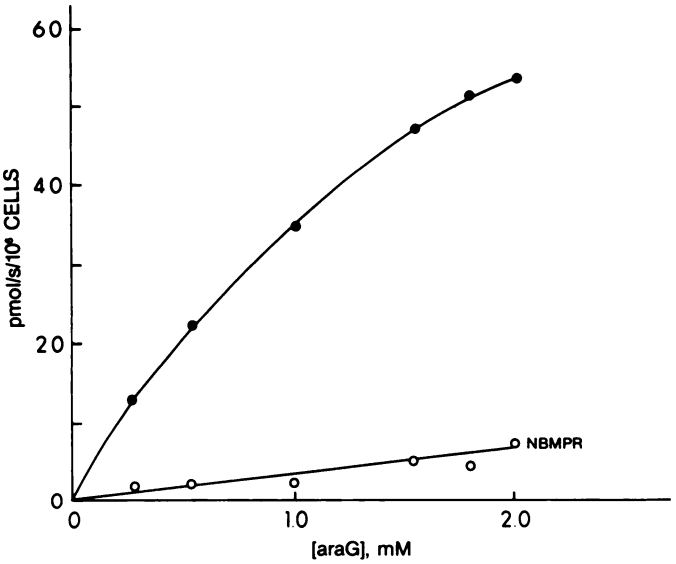

Fig. 5. Concentration dependence of [^{3}H]ara-G influx into MOLT 4 cells. ara-G (●); ara-G + 2.5 μM NBMPR (○). The initial velocity range for ara-G influx was 0–3 s.

cold papaverine was added first, followed by [^{3}H]ara-G ("zero time"), and the assay tubes were held for 15 to 30 s before centrifugation. Neither the assays that were stopped after 2 s nor those to which papaverine was added prior to addition of [^{3}H]ara-G yielded detectable rates, indicating that no further influx of permeant occurred after the addition of papaverine. The similarity of the influx rate of [^{3}H]ara-G obtained with an "oil stop" assay and the control rate determined with the use of cold papaverine provides further evidence that the stop agent is adequate. Moreover, cell-associated radioactivity determined at zero time was approximately equal to the extracellular space as measured with [^{14}C]sucrose. The use of papaverine was validated for each permeant and each cell type (data not shown).

Influx of 2.0 μM [^{3}H]ara-G into MOLT 4 cells was linear in the range of 0–3 s (Fig. 4). NBMPR (2.5 μM) inhibited ara-G influx although the apparent degree of inhibition decreased with increase in time of incubation with ara-G. The plot of initial velocity *versus* ara-G concentration tended toward saturability, but saturation could not be determined with certainty due to the limited solubility of ara-G (Fig. 5). The addition of 2.5 μM NBMPR revealed the presence of a second influx component representing 10–20% of the total influx rate. An apparent K_m of 1.7 ± 0.3 (SD) mM and a V_{max} of 92 ± 9 pmol/s/10^6 cells for influx (NBMPR-sensitive component) were obtained by subtracting the NBMPR-insensitive rate from the total influx. Due to experimental limitations, a K_m for the second transport system could not be obtained. However, the linearity to 2 mM of the *v versus* [*S*] plot indicates that $K_m >>$ 2 mM. In contrast, the influx of [^{3}H]tubercidin into MOLT 4 cells was clearly a saturable process (K_m 106 ± 12 μM, V_{max} 45 ± 2 pmol/s/10^6 cells) and very little NBMPR-insensitive transport was evident (Fig. 6).

Reversed-phase HPLC analysis of extracts of MOLT 4 cells incubated with [^{3}H]ara-G for 5 s indicated that 90% of the total cell-associated radioactivity was unmetabolized ara-G.

Effects of Nucleosides, Nucleobases, and Nucleoside Transport Inhibitors. Table 2 summarizes the data concerning the effects of various compounds on the influx of 10 μM [^{3}H]ara-G into MOLT 4 cells. The initial rate of influx was inhibited 90–93% by NBMPR, dilazep, and dipyridamole and 67–81% by various nucleosides. Nucleobases had no significant effect on the influx of ara-G when measured in the absence of NBMPR. However, 1.0 mM adenine or hypoxanthine inhibited the NBMPR-insensitive influx of 10 μM ara-G by 73 and 58%,

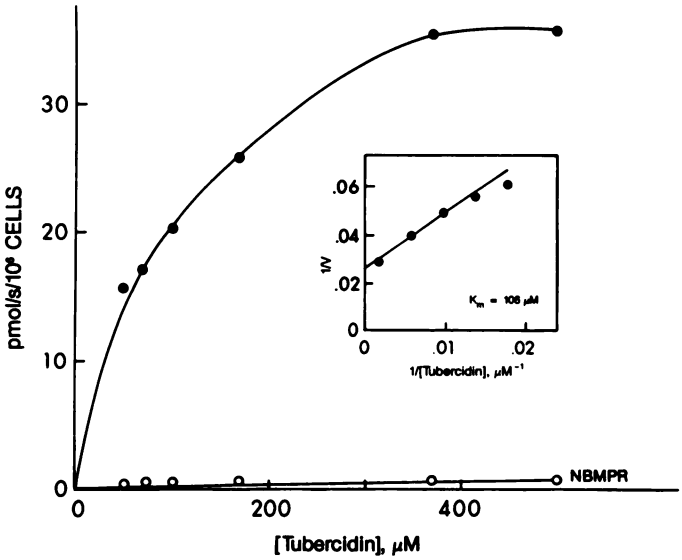

Fig. 6. Concentration dependence of [^{3}H]tubercidin influx into MOLT 4 cells. Tubercidin (●); tubercidin + 2.5 μM NBMPR (○). *Inset*, secondary plot of initial velocities of tubercidin influx determined at varying concentrations of tubercidin.

Table 2 *Effects of nucleobases, nucleoside transport inhibitors, and nucleosides on the influx of 10 μM [^{3}H]ara-G into MOLT 4 cells*

Initial velocities of the influx of [^{3}H]ara-G, with and without 2.5 μM NBMPR, were determined in the presence and absence of various agents.

Agent	% inhibition	
	Of control rate	Of NBMPR-inhibited rate
Adenine		
100 μM	0	ND[a]
1.0 mM	20	58
Hypoxanthine		
100 μM	0	ND[a]
1.0 mM	20	73
Dilazep, 2.5 μM	93	6
Dipyridamole, 5.0 μM	90	13
Guanosine, 1.0 mM	67	7
Inosine, 1.0 mM	81	0
Uridine, 1.0 mM	72	5

[a] ND, not determined.

respectively; whereas dipyridamole, dilazep, and various nucleosides had no further effect on influx in the presence of 2.5 μM NBMPR.

Intracellular Formation of 5'-Triphosphates. Extracts of MOLT 4 cells that had been incubated in the presence of 2.0 μM [^{3}H]ara-G for 24 h contained 0.85 pmol ara-GTP/10^6 cells, as determined by anion-exchange HPLC (Table 3). Addition of 5.0 μM NBMPR did not reduce the intracellular formation of ara-GTP. In contrast, the amount of tubercidin 5'-triphosphate formed from 2.0 μM [^{3}H]tubercidin in MOLT 4 cells under identical conditions was reduced by 80% when 5.0 μM NBMPR was included in the incubation medium.

Influx of [^{3}H]ara-G into Human Erythrocytes. NBMPR (2.5 μM) appeared to almost completely prevent the influx of 2.0 μM ara-G into human erythrocytes. Only a very low residual rate could be detected at high concentrations of ara-G in the presence of NBMPR (Fig. 7). The limited solubility of ara-G precluded precise kinetic analyses above 2.0 mM ara-G; an apparent K_m of 3.4 ± 0.1 mM and a V_{max} of 164 ± 4 pmol/s/5 μl packed cells were obtained.

DISCUSSION

Evidence has been presented which suggests that ara-G permeates the membrane of MOLT 4 cells by means of two distinct carrier-mediated systems: a NBMPR-sensitive nucleoside transporter; and a NBMPR-insensitive, nucleobase-inhibited transporter. The latter component mediates 10–20% of the total influx of this permeant in these cells. When analyzed kinetically, an apparent K_m of 1.7 mM was obtained for that component of ara-G transport which is mediated by the NBMPR-sensitive nucleoside carrier. The K_m for the NBMPR-insensitive component is much greater than 2 mM, as indicated by the linear dependency of velocity on ara-G concentration. The permeation efficiency (V_{max}/K_m) via the nucleoside transporter is 8-fold higher for tubercidin than for ara-G, indicating that tubercidin is a much better permeant than ara-G for the NBMPR-sensitive nucleoside transport system. The efficiency of ara-G may be greater than that of tubercidin for the NBMPR-insensitive component of transport in MOLT 4 cells, thus accounting for the measurable rate of ara-G influx. Multiple nucleoside transport systems within a single cell type have been described in the literature. NBMPR-sensitive and -insensitive uridine transporters have been reported to occur in L1210, RPMI 6410, L5178Y, and P388 cells (11, 12). NBMPR-insensitive nucleoside transport has also been demonstrated in transformed hamster fibroblasts (13, 14), Chinese hamster ovary cells (15), and HeLa cells (16). Tubercidin toxicity has been found to be abrogated by NBMPR in human peripheral blood mononuclear cells but not in human neuroblastoma cell lines (17), suggesting the presence of a NBMPR-insensitive influx process in the latter cell line.

Table 3 *Effects of NBMPR on the intracellular formation of ara-GTP or tubercidin 5'-triphosphate in MOLT 4 cells*

MOLT 4 cells (2×10^6 cells in 50 ml) were incubated for 24 h at 37°C in the presence of 2.0 μM [^{3}H]tubercidin or 2.0 μM [^{3}H]ara-G, with or without 5.0 μM NBMPR. Acid-soluble extracts of cells were analyzed by anion-exchange HPLC.

Agent	pmol/10^6 cells[a]	
	ara-GTP	Tubercidin 5'-triphosphate
Tubercidin		63.6 ± 6.6
Tubercidin + NBMPR		12.8 ± 0.5
ara-G	0.85 ± 0.07	
ara-G + NBMPR	0.85 ± 0.09	

[a] Mean ± range of two determinations.

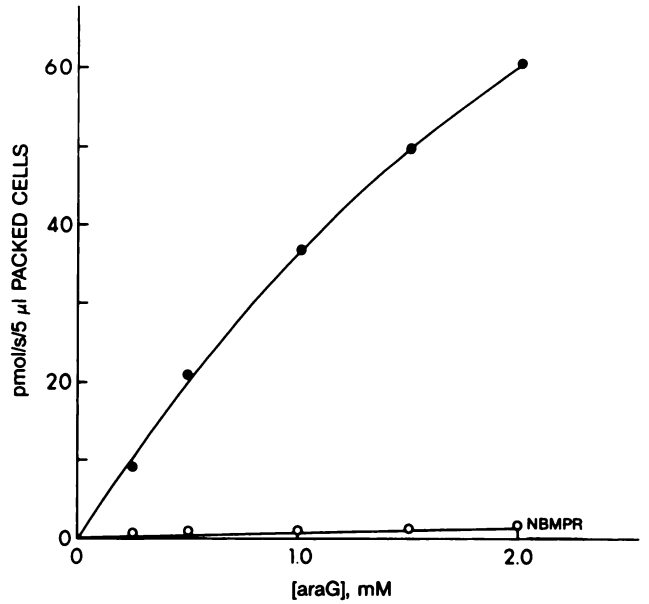

Fig. 7. Concentration dependence of [^{3}H]ara-G influx into human erythrocytes. ara-G (●); ara-G + 2.5 μM NBMPR (○).

The existence of significant multiple-transport systems for ara-G, but not for tubercidin, in MOLT 4 cells may explain the inability of NBMPR to reverse ara-G-induced growth inhibition. In contrast, both tubercidin-induced cytotoxicity to MOLT 4 cells and tubercidin influx into these cells were reversed by NBMPR. The metabolic data corroborate these observations, as the intracellular accumulation of tubercidin 5'-triphosphate from tubercidin was reduced by 80% in the presence of NBMPR, whereas the intracellular accumulation of ara-GTP from ara-G remained the same in the presence or absence of NBMPR. Since less than 2 pmol ara-GTP/10^6 cells has been found to inhibit DNA synthesis (1), the ara-G supplied to the cell by the alternate transport system may be entirely sufficient to exert the cytotoxic effect.

The transport characteristics of CCRF CEM cells with respect to ara-G and tubercidin were not investigated directly. However, the similar abilities of NBMPR, dilazep, and dipyridamole to protect these cells against growth inhibition by tubercidin but not by ara-G indicates that they may, like MOLT 4 cells, also possess a NBMPR-insensitive influx mechanism for ara-G.

The multiplicity of transport systems for ara-G appears to be a characteristic of T-lymphoblastoid cell lines, since essentially all the transport activity in human erythrocytes can be accounted for by the NBMPR-sensitive nucleoside transport system. The ability of ara-G to utilize two transport systems in human T-cell leukemia lines may offer a chemotherapeutic advantage in the selective treatment of human T-cell leukemias and concurrent protection of normal cells by inhibitors of nucleoside transport.

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